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COMPARISON OF CERTAIN LABORATORY TESTS IN THE DIAGNOSIS OF HASHIMOTO'S THYROIDITIS*

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ABSTRACT

A lowered level of serum albumin and an increased PBI-BEI difference have diagnostic value in Hashimoto's thyroiditis. The zinc sulfate concentration and the erythrocyte sedimentation rate were abnormally high in a large proportion of 101 patients having Hashimoto's thyroiditis, but also in nearly as high a proportion of 39 euthyroid patients with nodular goiters. For this reason the practical diagnostic usefulness of these two tests is negligible. When the zinc sulfate, PBI-BEI difference, alpha-1-globulin, and alpha-2-globulin tests were considered as a group, one or more gave abnormal results in 97 per cent of patients with thyroiditis and in 72 per cent of euthyroid patients with adenomatous goiter. This surprising proportion of abnormal values in the cases of euthyroid adenomatous goiter makes the use of such combinations of tests of relatively little value in the diagnosis of Hashimoto's thyroiditis. It is not known why so many of these laboratory findings are abnormal in patients with Hashimoto's thyroiditis and in an equally impressive, although significantly smaller proportion, of euthyroid patients with adenomatous goiters.

BECAUSE of the increasing incidence of Hashimoto's thyroiditis, laboratory procedures of diagnostic value for this disorder are becoming more important. In 1954 Cooke and Wilder (1) reported persistently ab-

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TABLE 1. NORMALITY OF FINDINGS FROM 11 DIAGNOSTIC LABORATORY TESTS IN 101 PATIENTS WITH HASHIMOTO'S THYROIDITIS AND 39 EUTHYROID PATIENTS WITH ADENOMATOUS GOITER

Test	Normal range*	Abnormal values (% of cases)	
		Hashimoto's thyroiditis	Euthyroid, with adenomatous goiter
Erythrocyte sedimentation rate, mm./hr. (Westergren)	0–20	49	38
Thymol turbidity, units	0–3	11	26
Cephalin-cholesterol flocculation, grade	0–2	34	10
Zinc sulfate, units	0–18	60	49
Total protein, Gm./100 ml. serum	6.0–7.7	2	5
Albumin	3.3–4.3	51	28
Alpha-1 globulin	0.3–0.4	37	28
Alpha-2 globulin	0.5–0.8	37	24
Beta globulin	0.6–1.1	3	8
Gamma globulin	0.8–1.6	21	5
PBI-BEI difference, μg./100 ml. serum	0–1.0	47	10

* As determined by statistical analysis in the laboratories of the Mayo Clinic.

normal results of the serum colloidal gold test in patients who had Hashimoto's thyroiditis, but not in patients who had spontaneous myxedema not caused by this disorder. The next year Cooke and Luxton (2) reported that results of the thymol turbidity test as well as the colloidal gold test were abnormal in many cases of Hashimoto's thyroiditis. In 1956 Skillern and associates (3) reported from a study of 36 patients with this disease that serum albumin and alpha globulin levels were decreased in many instances, beta and gamma globulin levels were increased, and results of the colloidal gold, thymol turbidity, zinc sulfate and cephalin-cholesterol flocculation tests were abnormal. These workers also commented on the abnormally large difference between the values for serum protein-bound iodine (PBI) and butanol-extractable iodine (BEI) in some patients with Hashimoto's thyroiditis. This abnormal PBI-BEI difference had been noted previously by Gribetz and associates (4) in several young girls with struma lymphomatosa.

We undertook this study to evaluate critically such laboratory procedures in a large number of patients with proved Hashimoto's thyroiditis.

MATERIALS AND METHODS

From 101 patients with Hashimoto's thyroiditis proved histologically (with thyroidectomy or needle biopsy) a compilation was made of findings with respect to the erythrocyte sedimentation rate (Westergren method), thymol turbidity test, cephalin-cholesterol flocculation test, zinc sulfate test, serum protein electrophoresis—including

determination of total proteins, albumin, and alpha-1, alpha-2, beta, and gamma globulins—and the difference between the values for the serum PBI and BEI (Table 1).

The serum protein studies were performed by a paper electrophoretic method based on that of Durrum (5), and the protein fractions were quantitated densitometrically after staining with amidoschwarz. The concentration of serum protein-bound iodine was determined by a modification of the Barker dry-ash technic (6), and that of butanol-extractable iodine by subtracting the butanol-insoluble iodine from the protein-bound iodine.

Control values for these procedures were determined on the sera of 39 euthyroid patients with adenomatous goiter proved by thyroidectomy.

RESULTS

As seen in Table 1, no procedure tested is sufficiently discriminating to be diagnostically useful alone. The tests that most frequently yielded abnormal data for the 101 patients having Hashimoto's thyroiditis were: zinc sulfate, with results abnormally high in 60 per cent of cases; serum albumin, abnormally low in 51 per cent; erythrocyte sedimentation rate, abnormally high in 49 per cent; and PBI-BEI difference, abnormally high in 47 per cent. To our surprise, however, the values from the zinc sulfate test and the sedimentation rate were likewise abnormally high and the serum

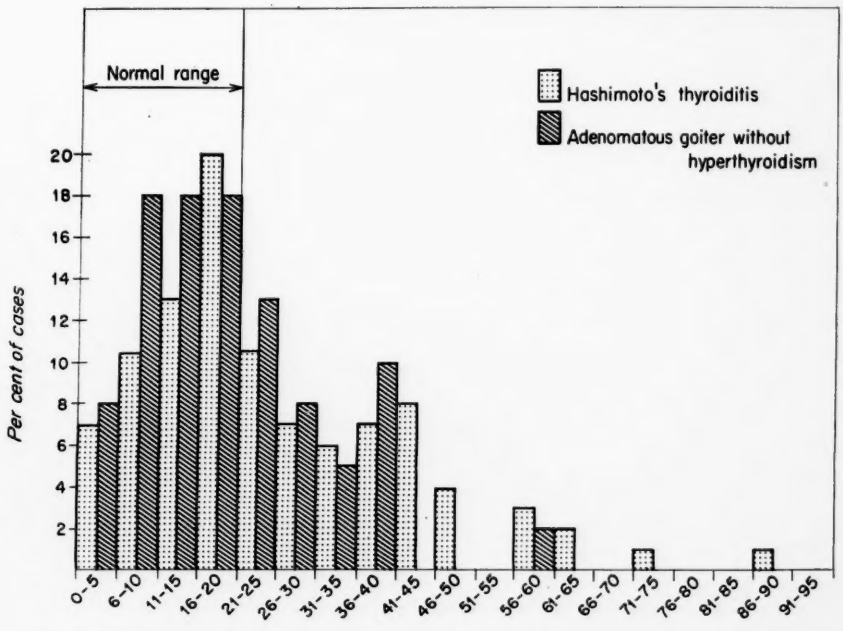

FIG. 1. Erythrocyte sedimentation rate (mm. per hr.) (Westergren). Comparison of percentage distributions in cases of Hashimoto's thyroiditis and cases of adenomatous goiter without hyperthyroidism.

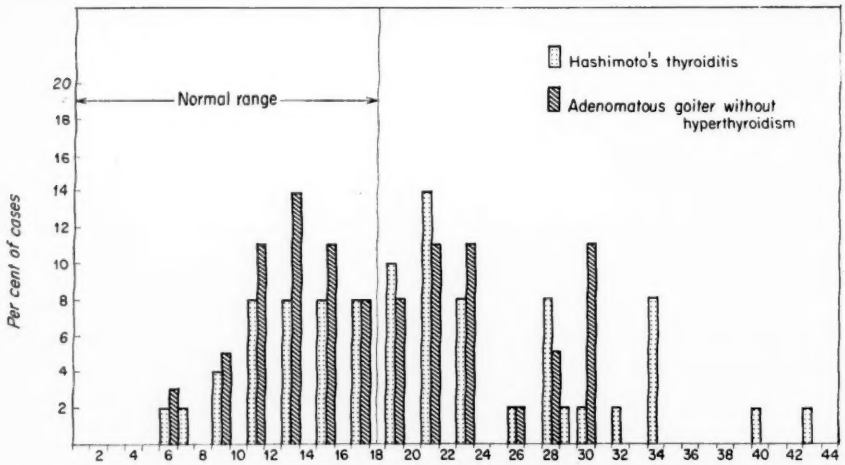

FIG. 2. Zinc sulfate flocculation (units). Comparison of percentage distributions in cases of Hashimoto's thyroiditis and cases of adenomatous goiter without hyperthyroidism.

albumin level was likewise abnormally low in a significant number of euthyroid patients with adenomatous goiter.

The erythrocyte sedimentation rate by itself is not a reliable indicator of Hashimoto's thyroiditis (Fig. 1). Although the sedimentation rate in the patients with this disorder whom we studied was sometimes very high (above 40 mm. per hour in 19 per cent) and was above the normal range in 49 per cent, the overlap with the values for the control group was too large for this test to be of diagnostic value when used alone. In addition, myxedema itself is known to be associated frequently with an elevated erythrocyte sedimentation rate (7, 8). Twenty-one of the 101 patients with Hashimoto's thyroiditis had myxedema; and sedimentation rates, determined for 19 of them, were abnormally high in 68 per cent. This observation detracts further from the diagnostic usefulness of the test.

As shown in Figure 2, results of zinc sulfate tests were abnormally high in 60 per cent of patients with Hashimoto's thyroiditis. Unfortunately, this finding is without specific diagnostic value, since in nearly as many of the control cases (49 per cent) the values were also abnormally high.

Somewhat better separation between the two groups was obtained by measurement of the serum albumin level (Fig. 3). Values for serum albumin were below the normal range in 51 per cent of the patients with Hashimoto's thyroiditis, but in only 28 per cent of control patients.

The single laboratory finding which best differentiated Hashimoto's thyroiditis from adenomatous goiter without hyperthyroidism was the disparity between serum protein-bound iodine and butanol-extractable iodine

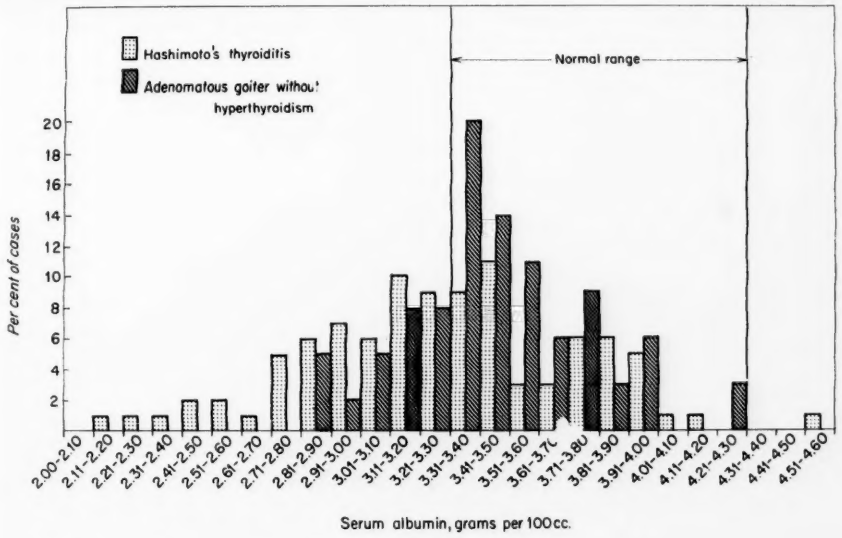

FIG. 3. Serum albumin (Gm. per 100 ml.). Comparison of percentage distributions in cases of Hashimoto's thyroiditis and cases of adenomatous goiter without hyperthyroidism.

(Fig. 4). In 47 per cent of the patients who had Hashimoto's thyroiditis the PBI-BEI difference exceeded the normal limit of 1.0 μg. per 100 ml., and in 36 per cent it was more than 2.0 μg. Among the euthyroid patients with adenomatous goiter this difference was greater than 1.0 μg. in only 10 per cent of cases, and in none was it as great as 2.0 μg.

When the results from two, three or four procedures are grouped together, certain combinations might be expected to increase the diagnostic accuracy of the procedures; but actually this was of little help. When findings from the zinc sulfate, PBI-BEI difference, alpha-1 globulin, and alpha-2 globulin tests were considered as a group, one or more gave abnormal results in 97 per cent of patients with Hashimoto's thyroiditis and in 72 per cent of the euthyroid patients with adenomatous goiters. This high proportion of abnormal results in the cases of euthyroid adenomatous goiter makes the use of such combinations of tests of relatively little value in the diagnosis of Hashimoto's thyroiditis.

COMMENT

These findings are in substantial agreement with those published by Luxton and Cooke (9) and Skillern and associates (3), despite some exceptions. We did not find the serum albumin level usually normal in patients with Hashimoto's thyroiditis, as did Luxton and Cooke, but frequently

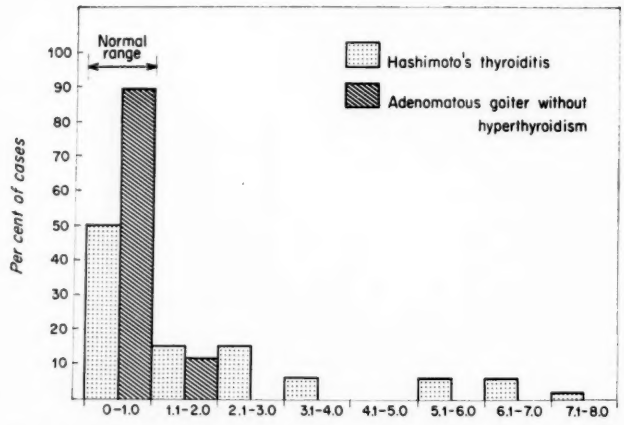

FIG. 4. Difference between serum protein-bound iodine and butanol-extractable iodine (PBI–BEI difference) (μg. per 100 ml.). Comparison of percentage distributions in cases of Hashimoto's thyroiditis and cases of adenomatous goiter without hyperthyroidism.

found it abnormally low. Skillern and associates used Longworth's modification of the Tiselius technic, and reported that the quantity of alpha-2 globulin was often low, and the thymol turbidity value was more often abnormally high than was the zinc sulfate test value. On the contrary, we found the concentration of alpha-2 globulin frequently to be abnormally high—rarely low—and the results of the zinc sulfate test to be abnormally high far more often than those of the thymol turbidity test.

Since myxedema frequently develops in patients with Hashimoto's thyroiditis, we considered the possibility that myxedema itself is a factor in the appearance of these abnormal test results. However, this does not seem to be so, except with regard to the erythrocyte sedimentation rate and perhaps the zinc sulfate test. Among 6 patients with myxedema not caused by Hashimoto's thyroiditis (Table 2), the sedimentation rate was elevated in 5, and the zinc sulfate result in 4; but thymol turbidity, cephalin-cholesterol flocculation, and the electrophoretic pattern were mostly within the normal range. Since in myxedema both PBI and BEI levels are very low, the PBI-BEI difference has no significance.

It is not known why these laboratory findings are sometimes abnormal in cases of Hashimoto's thyroiditis and adenomatous goiter. Luxton and Cooke (9) suggested that in Hashimoto's thyroiditis the diseased thyroid may produce some abnormal secretion which damages the reticulo-endothelial system as a whole. They advanced this hypothesis because they found enlargement of the spleen in 6 of 24 cases of Hashimoto's thyroiditis

and an abnormal histologic picture in 2 of 3 livers (biopsy specimens) in the same series. McConkey and Callaghan (10) reported 3 patients who had Hashimoto's thyroiditis and diffuse hepatic fibrosis, and suggested an association between these two diseases. However, neither overt liver disease nor splenomegaly was a significant factor in our series of 101 patients with Hashimoto's thyroiditis.

It has been shown by Owen and McConahey (11) that the serum of many patients with this disorder contains varying quantities of abnormal circulating "thyroglobulin." It is this excess thyroglobulin which accounts for the abnormal PBI-BEI difference and may account for some of the other abnormal test findings.

Since it has been demonstrated that serum albumin is an anti-flocculation agent (12), perhaps it was the low level of albumin in the serum which caused the abnormal flocculation values in some of our patients, although there appeared to be no correlation between the positive flocculation results and the albumin values in patients with either thyroiditis or adenomatous goiter. Since gamma globulin is a flocculating agent (13), perhaps the elevated concentrations of gamma globulin in some patients with Hashimoto's thyroiditis are a factor contributing to the abnormality of the flocculation results. There is some evidence that alterations in the lipoprotein pattern may play an important part in positive flocculation results seen in a variety of diseases (14). So far as we know, lipoproteins have not been studied in Hashimoto's thyroiditis.

TABLE 2. RESULTS FROM 11 DIAGNOSTIC LABORATORY TESTS IN 6 CASES OF MYXEDEMA*

Tests†	Cases					
	1	2	3	4	5	6
Erythrocyte sedimentation rate	31‡	31‡	45‡	22‡	10	37‡
Thymol turbidity	2	—	3	6‡	2	4‡
Cephalin-cholesterol flocculation	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Zinc sulfate	19‡	21‡	19‡	40‡	13	15
Total proteins	6.82	6.82	7.26	7.86‡	6.71	6.60
Serum albumin	3.64	3.51	3.68	3.73	3.81	3.22‡
Alpha-1 globulin	0.32	0.33	0.43‡	0.34	0.31	0.32
Alpha-2 globulin	0.68	0.77	0.72	0.60	0.48‡	0.58
Beta globulin	1.03	1.00	1.12‡	1.01	0.97	0.95
Gamma globulin	1.15	1.21	1.31	2.18‡	1.14	1.53
PBI-BEI difference	—	0.4	0	—	—	0

* Case 1, post-I^{131}; all others, idiopathic.
† For units of quantitation and normal range, see Table 1.
‡ Abnormal values.

We have no explanation for the significant number of abnormal results obtained with these laboratory procedures in euthyroid persons with adenomatous goiters.

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STUDIES OF PENTOSE METABOLISM IN NORMAL SUBJECTS AND IN PATIENTS WITH PENTOSURIA AND PENTOSURIA TRAIT*

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ABSTRACT

Endogenous serum L-xylulose concentration exceeds 1 mg. per 100 ml. in pentosuric patients, and the level rises several-fold following administration of glucuronolactone. L-xylulose is not detectable in the serum of most nonpentosuric subjects before administration of glucuronolactone, but appears following ingestion of this compound. The increase in both serum and urine pentose levels after glucuronolactone is greater in patients with pentosuria trait than in control subjects. A comparison of serum and urine levels suggests that both pentosuric and nonpentosuric subjects excrete most of the L-xylulose which is presented to the renal tubules. TPN-xylitol (L-xylulose) dehydrogenase, the enzyme believed to be defective in subjects with pentosuria, can be demonstrated in normal human liver and in rat and guinea-pig liver and kidney.

PENTOSURIA is an inborn error of metabolism, which is characterized by the urinary excretion of 1–4 grams of L-xylulose per day. There is now overwhelming evidence (2, 3) that this condition is attributable to a block in the reaction in the glucuronic-acid oxidation pathway which results in the conversion of L-xylulose to xylitol. We have measured the level of the enzyme which catalyzes this reaction in several tissues from nonpentosuric human subjects, rats, and guinea pigs. In addition, we have used a sensitive and specific enzymatic assay to investigate levels of L-xylulose in the serum and urine of patients with pentosuria or pentosuria trait, and of several control subjects. In this paper we present these data and consider their implications with respect to the nature and the transmission of pentosuria.

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A preliminary report of a portion of these data has been presented (1).

METHODS

Subjects and procedure

The studies of serum and urine L-xylulose levels were carried out with the subjects in the postabsorptive state, in 3 patients with pentosuria, 5 close relatives of pentosurics, and 25 persons selected from hospital patients and laboratory personnel. The control subjects are listed in Table 1 according to their religious background, for pentosuria has been described almost exclusively in Jews. The heterozygous subjects included 3 parents of pentosurics (#4, #5 and #7 in Table 1), the son of a pentosuric mother (#8), and one (#6) of 5 siblings, 3 of whom had pentosuria. The patients with liver disease were made available for study through the courtesy of Dr. Thomas Chalmers, Lemuel Shattuck Hospital. Control Subject #19 had severe untreated hyperthyroidism.

Venous blood and, in some instances, urine samples were obtained before and at intervals after the subjects drank about 400 ml. of water containing glucuronolactone. Urine and serum were kept in ice and either assayed within two hours of the study, or stored at −20° C. and assayed within forty-eight hours.

Assay of L-xylulose

A 2-ml. sample of serum was deproteinized with 0.2 ml. of 70 per cent perchloric acid and centrifuged; 1 ml. of the supernatant solution was brought to pH 7.2 with 2 *N* potassium hydroxide. The insoluble potassium perchlorate was removed by centrifugation at 20° C., and an aliquot of the clear supernatant solution was used in the assay. Urine samples were adjusted to pH 7.2 prior to analysis. The assay mixture included 40 μmoles of tris(hydroxymethyl)aminomethane (Tris) maleate, pH 7.5; 5 μmoles of magnesium chloride; 1 μmole of cysteine; 0.1 μmole of reduced triphosphopyridine nucleotide (TPNH); urine or deproteinized serum; and an excess of the ammonium sulfate fraction of the guinea-pig liver TPN-xylitol (L-xylulose) dehydrogenase described by Hickman and Ashwell (4) in a final volume of 1.0 ml. The reaction was begun by the addition of enzyme and was followed spectrophotometrically at 340 mμ. With the enzyme preparation used, no significant reaction occurred with D-glucuronolactone, D-glucuronic acid, L-gulonolactone, or D-xylulose.

Assay of TPN-xylitol (L-xylulose) and DPN-xylitol (D-xylulose) dehydrogenases

Human liver biopsy specimens were obtained during surgery for biliary-tract disease from patients (#34–#39, Table 4) receiving general anesthesia. The tissue samples were kept in ice until homogenized, usually within thirty minutes of removal. Most biopsy specimens were not examined histologically. However, all but one appeared grossly normal; in Patient #39 there was both histologic and functional evidence of alcoholic cirrhosis.

Male guinea pigs each weighing 300–450 Gm. and male Wistar rats each weighing 250–300 Gm. were sacrificed by decapitation, and the organs to be studied were quickly removed and placed in ice.

Human liver was disrupted with 10 volumes, and the rat tissues with 5 volumes of ice-cold 0.25 *M* sucrose for one minute in a Teflon-glass homogenizer. The extracts were centrifuged at 0° C. for one hour at 30,000×g, and the supernatant solution was used for the enzyme assays. Human leukocytes were isolated by a modification (5) of the method of Skoog and Beck (6) and disrupted by sonication (5).

TPN-xylitol dehydrogenase was assayed by adding tissue extract to a 1-ml. cuvette containing 90 μmoles of Tris buffer, pH 7.5; 4 μmoles of magnesium chloride; 0.8 μmole of cysteine; 0.25 μmole of L-xylulose; and 0.11 μmole of TPNH in a final volume of 1.0

ml. Oxidation of TPNH was followed by measuring spectrophotometrically the fall in optical density at 340 mμ. DPN-xylitol dehydrogenase was assayed similarly, except that D-xylulose and reduced diphosphopyridine nucleotide (DPNH) were substituted for L-xylulose and TPNH, respectively. The specific activity of both dehydrogenases is expressed as the change in optical density at 340 mμ per minute in 1 ml. of reaction mixture per mg. of protein, measured by the biuret procedure (7).

Materials

D-glucuronolactone, D-glucuronic acid, and L-gulonolactone were purchased from Nutritional Biochemicals Corporation, and TPNH and DPNH were products of Sigma Chemical Company. The D-xylulose was a generous gift from Dr. Gilbert Ashwell, and

TABLE 1. SERUM L-XYLULOSE LEVELS AFTER ADMINISTRATION OF GLUCURONOLACTONE

Subjects	Glucurono-lactone (Gm.)	Serum L-xylulose (mg./100 ml.) minutes after glucuronolactone						
		0	30	60	90	120	150	180
Pentosurics								
1	5	1.2	6.6	7.2	4.9	4.7	4.4	
2	10	1.3	7.8	9.9	9.9		9.7	10.0
3	10	1.7	14.7		11.9		9.0	
Heterozygotes								
4	25	*	3.9		3.4			1.2
5	25	0.17		1.3		0.54		
6	25	*	0.93		0.54			0.21
7	25	*	0.77	0.77		0.19		
8	25	*	1.41		1.01			0.64
Controls								
Non-Jewish								
9	5	*	*	*				
10	25	*	0.25					0.23
11	25	*	0.17					0.11
12	25	*	0.28		0.36			0.19
13	25	0.18	0.20		*	*		*
14	25	0.14	0.20		0.35	*		0.32
15	25	*	0.11			0.23		
16	25	*	0.19					
17	25	*	0.19		0.18		*	
18	25	*	0.19		0.11		*	
19 (hyperthyroid)	25	*	0.11	0.11				
Jewish								
20	25	*	0.20		0.24			
21	25	0.23	0.52		0.41			
22	25	*	0.18		0.19		*	
23	25	*	0.14		0.29		0.20	
24	25	*	0.27		0.25		*	
25	25	*	0.22		0.28		0.18	
26	25	*	0.19		0.17		0.12	
27	25	*		0.45			0.14	
Liver disease (non-Jewish except #30)								
28. Alcoholic cirrhosis	25	*	0.28		0.51		0.11	
29. Biliary cirrhosis	25	*	0.24		0.10			
30. Post-necrotic cirrhosis	25	0.21			0.55			
31. Alcoholic cirrhosis	25	0.15	0.30		0.40		0.20	
32. Alcoholic cirrhosis	25	0.11			0.26		0.21	
33. Alcoholic cirrhosis	25		0.27			0.47		

* Less than 0.10 mg. per 100 ml.

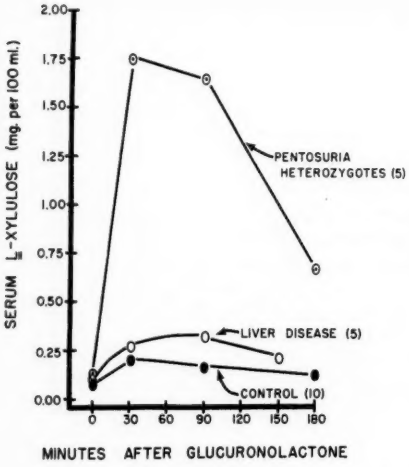

FIG. 1. Average serum L-xylulose levels before and after administration of glucuronolactone in 10 non-Jewish control subjects, 5 subjects heterozygous for pentosuria, and 5 patients with severe, chronic liver disease.

L-xylulose was isolated from pentosuric urine by the procedure described by Touster *et al.* (8). During the isolation procedure, chemically synthesized L-xylulose (kindly provided by Dr. Ashwell) was used as a marker.

RESULTS

Serum and urine L-xylulose

Serum L-xylulose levels (fasting) in excess of 1 mg. per 100 ml. were found in all 3 pentosuric patients (Table 1). In contrast, no L-xylulose could be detected in the sera of most other subjects. The ingestion of glucuronolactone was followed in almost all cases by a rise in the level of serum L-xylulose, with peaks occurring at thirty to ninety minutes. The levels of serum pentose were very much higher following glucuronolactone in the pentosuric relatives than in the controls. This difference is shown graphically in Figure 1, in which average serum values are plotted for the pentosuric heterozygotes and for the non-Jewish control subjects. Of particular note is the fact that peak values in subjects with pentosuria trait varied from 0.77 to 3.9 mg. per 100 ml., whereas peak values in the non-Jewish control group did not exceed 0.36 mg. per 100 ml. Also shown in Figure 1 are the average serum pentose levels in the 5 non-Jewish (#30 was Jewish) patients with liver disease.

Differences in urinary pentose excretion (Table 2) as well as in serum pentose levels (Table 1) distinguish the pentosurics, the subjects with pentosuria trait, and the control group. The values for glomerular filtration rates shown in Table 3 were calculated from endogenous creatinine levels and, as such, are only gross approximations. However, they do permit estimates which indicate that much, if not all of the filtered L-xylulose appeared in the urine of subjects with pentosuria and pentosuria trait.

TABLE 2. RATE OF URINARY EXCRETION OF L-XYLULOSE AFTER ADMINISTRATION OF GLUCURONOLACTONE

Subjects	Glucuronolactone (Gm.)	Urine L-xylulose (mg./hr.) hours after glucuronolactone			
		0	1	2	4
Pentosurics					
1	5		304	320	
2	10	106	236	549	200
3	10	88	271	451	
Heterozygotes					
6	25	0	81	54	6
8	25	0.1	63	31	20
Controls					
10	25	0	9	10	3
11	25	0	23	18	3
12	25	0	17	12	5
13	25	0.6	13	18	6
19 (hyperthyroid)	25	0	5	5	

Similarly, if average glomerular filtration rates are assumed for the control subjects whose serum and urinary levels are listed in Tables 1 and 2, one can calculate that in this group, too, most of the filtered L-xylulose was excreted.

Enzyme levels in tissues

A deficiency of TPN-xylitol (L-xylulose) dehydrogenase, the enzyme which catalyzes the conversion of L-xylulose to xylitol, is believed to be the lesion in pentosuria (2, 3). Since the enzymatic block has yet to be demonstrated directly, a search was made for this enzyme in readily available human tissues and secretions. However, no evidence of dehydrogenase activity was found in leukocytes, erythrocytes, saliva, semen, or adipose tissue of normal human subjects. On the other hand, substantial activity was found in liver biopsy specimens from several nonpentosuric subjects, and these data are presented in Table 4. Comparable levels of activity were observed in rat liver and kidney, and considerably higher levels in guinea-

TABLE 3. RENAL CLEARANCE OF L-XYLULOSE BY SUBJECTS WITH PENTOSURIA AND PENTOSURIA TRAIT

Subjects	Total concurrent time (mins.)	Urine flow (ml./min.)	Creatinine clearance (ml/min.)	Plasma xylulose (μg/ml.)	Xylulose (mg./min.)			Per cent xylulose reabsorbed
					Filtered	Excreted	Reabsorbed	
2 (pentosuric)	0–60	1.33	137	17.4	2.38	2.60	0	0
	60–120	10.3	178	17.4	3.10	2.63	0.47	15
8 (pentosuric heterozygote)	0*–60	0.75	128	10.0	1.28	1.05	0.23	18
	60–120	0.83	74	10.1	0.74	0.52	0.22	30

* 25 Gm. of D-glucuronolactone given at 0′ to Subject #8; no glucuronolactone was given to Subject #2.

TABLE 4. TPN-XYLITOL (L-XYLULOSE) AND DPN-XYLITOL (D-XYLULOSE) DEHYDROGENASES IN MAMMALIAN TISSUES

Source	TPN-Xylitol dehydrogenase*		DPN-xylitol dehydrogenase*	
	Liver	Kidney	Liver	Kidney
Human subjects				
34	.057			
35	.072			
36	.071			
37	.064		.132	
38	.042		.151	
39**	.034		.075	
Average (excluding #39)	*.061*		*.119*	
Guinea pigs	(10 animals) .190–.554	.098–.298	(4 animals) .285–.408	.214–.260
Average	.378	.214	*.355*	*.239*
Rats	(15 animals) .061–.160	.048–.272	(5 animals) .216–.300	.220–.253
Average	.102	.122	*.242*	*.238*

* Changes in OD_{340} per min. per mg. protein per 1.0 ml.
** Alcoholic cirrhosis.

pig liver and kidney. Very low activity was found in rat spleen.

Evidence of DPN-xylitol (D-xylulose) dehydrogenase, the enzyme which mediates the interconversion of xylitol and D-xylulose, was found in human liver and in rat and guinea-pig liver and kidney (Table 4). The administration of chlorbutanol (100 mg. per day intraperitoneally for two days, or 60 mg. per day by mouth for five days) to rats and guinea pigs did not result in any change in activity of either the TPN-xylitol or the DPN-xylitol enzyme in liver or in kidney.

DISCUSSION

The evidence for a block in the glucuronic-acid oxidation pathway of carbohydrate metabolism in pentosuria, and the reactions involved in this scheme, are discussed in detail elsewhere (2, 3). The data described in this paper provide further support for a disturbance in the metabolism of glucuronic acid, and exclude the possibility (9) that a defect in renal tubular function accounts for the excretion of L-xylulose by pentosurics. Indeed, our data suggest that in both pentosuric and nonpentosuric subjects there is little, if any, tubular reabsorption of the pentose. Chromatographic technics have been used by others to demonstrate xylulose in pentosuric plasma, and to show an increase in this sugar following administration of glucuronolactone (10, 11). The data obtained with the sensitive and specific enzymatic technic used in these studies indicate that the xylulose is the L-form. (The chromatographic technics do not distinguish between the stereoisomers of the pentose.) Our studies also demonstrate that L-xylulose can be detected in small quantity in the sera of most normal persons following glucuronolactone loading. They also indicate that although subjects

heterozygous with respect to the pentosuria gene have no demonstrable xylulose in their serum or urine under resting conditions, considerable quantities, as compared to levels found in control subjects, appear following the "stress" of glucuronolactone administration.

The careful family studies of Lasker and her associates (12) demonstrate that pentosuria is transmitted as a simple autosomal character, and our studies are in accord with this conclusion. For example, in the 3 parents of pentosurics whom we tested (Subject 4 is the mother of Subject 2, and Subjects 5 and 7 are the parents of Subject 1), the pentose levels following administration of glucuronolactone were very much higher than those observed in the controls. In 2 of our Jewish "control" subjects, #21 and #27, the L-xylulose levels following glucuronolactone were in excess of the highest seen in the non-Jewish control group. This is of particular interest in Subject #27, since he was known to be distantly related to a pentosuric patient.

Although the serum L-xylulose levels were slightly elevated following administration of glucuronolactone in 3 of 5 non-Jewish subjects with severe liver disease, the differences from the levels in control subjects were not sufficient to warrant any conclusions. It is of interest that in Subject 19, who had severe untreated hyperthyroidism, there was no elevation of L-xylulose after glucuronolactone in either serum or urine, as compared with the euthyroid controls. This observation may be compared with a report that the administration of triiodothyronine to man (13) results in an increase in L-xylulose excretion.

A search for TPN-xylitol dehydrogenase was carried out in a variety of tissues and secretions from nonpentosuric human subjects in the hope that an opportunity would be provided for demonstrating readily the enzymatic defect in pentosuria. Of the human tissues examined, however, enzyme activity was found only in liver; and in the animal tissues examined, only in liver, kidney, and to a much lesser extent in spleen. It is noteworthy that no evidence of this enzyme was found in adipose tissue, particularly since growth hormone has been reported to effect an increase in the metabolism of the sixth carbon atom of glucose in epididymal fat (14); the increase has been interpreted as suggesting a stimulation of the glucuronic-acid oxidation pathway. Moreover, the administration of chlorbutanol, which stimulates the production of glucuronic acid in animals (15), did not change the activity of either dehydrogenase.

The higher levels of dehydrogenase activities in guinea-pig tissues as compared with rat tissues may be a reflection of differences in the metabolic pathways operative in these two species. In the rat, glucuronic acid may undergo either of two series of transformations—one leading to ascorbic acid, and the other through L-xylulose to hexose. In the guinea pig, on the other hand, only the second avenue is available (16, 17). Since man re-

sembles the guinea pig with respect to inability to synthesize ascorbic acid, we expected that the levels of the dehydrogenases in human liver might be closer to those observed in the guinea pig. Instead, lower levels were found. However, the studies in human subjects differed from those in animals, in that the human tissues were obtained at the time of surgery after the patients had received considerable preoperative and operative medication, whereas the animal tissues were obtained from rats and guinea pigs not subjected to such treatment. The effect of these medications on the activity of the dehydrogenases has not been ascertained.

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PERFUSION STUDIES OF THE HUMAN PLACENTA. II. METABOLISM OF C^{14}-17β-ESTRADIOL WITH AND WITHOUT ADDED HUMAN CHORIONIC GONADOTROPIN*†

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ABSTRACT

The metabolism of C^{14}-17β-estradiol was studied in the human placenta perfused *in vitro*. Four placentas were perfused for eight hours each with a change of perfusing fluid every hour. Perfusates for 15 of the 32 hours were analyzed individually for metabolites of the radioactive estradiol added at the beginning of the various hours. In addition, 1 placenta was subjected to extraction at the end of the perfusion. The radioactive metabolites were identified by paper chromatography, countercurrent distribution, and derivative formation. The possible effect of human chorionic gonadotropin (HCG) on the formation of metabolites was studied as follows: HCG was not added during perfusion I; HCG was added during each hour of perfusion II; in perfusions III and IV HCG was added only during the last five and four hours respectively and not during the first three and four hours. C^{14}-estriol was not found in the perfusates for hours 2, 4 and 7 respectively of perfusion I (no HCG). C^{14}-estriol was found in the perfusates for hours 2, 4 and 7 respectively of perfusion II (with HCG). In perfusion III no C^{14}-estriol was found in the perfusate for hour 2 (no HCG), but it was identified in the perfusates for hours 4, 6 and 8 respectively (with HCG). No C^{14}-estriol was found for hour 4 (no HCG) or hours 7 and 8 respectively (with HCG) of perfusion IV. C^{14}-estriol was also found in the extract of the placenta from perfusion III. The proportion of radioactivity recovered as C^{14}-estriol ranged from 0.7 to 4.5 per cent. These experiments demonstrate that the human placenta is capable of converting estradiol to estriol. The conversion has thus far been observed only when HCG was added to the perfusing fluid. This apparent effect of HCG on estrogen metabolism points to a possible role of HCG in placental self-regulation. C^{14}-2-methoxyestrone was tentatively identified in the perfusates for hour 4 of perfusion II, in the perfusates for hours 4, 6 and 8 respectively of perfusion III, and in the extract of the placenta from perfusion III (0.7 to 2.0 per cent). C^{14}-16-epiestriol was tenta-

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tively identified in the perfusate for hour 4 of perfusion II and in the extract of the placenta from perfusion III (about 1 per cent of recovered radioactivity). C^{14}-estrone was identified in each perfusate (58–82 per cent).

ALTHOUGH the production of estrogen by the placenta during human pregnancy has been clearly established (see review in (2)), the extent of the participation of the placenta in estrogen metabolism is as yet unknown. The demonstration that the intact human placenta obtained at term and then perfused *in vitro* remains metabolically active (3) suggested the present more direct approach to the problem of estrogen metabolism by the placenta. This perfusion technique has been used for the study of steroid synthesis and metabolism; the results obtained by perfusion of radioactive 17β-estradiol through the placenta are presented in this paper. The effect of human chorionic gonadotropin (HCG) on estrogen metabolism in such a perfusion was studied. Metabolites of estradiol not previously demonstrated as produced by the placenta have been found.

METHODS AND MATERIALS

Placental perfusion

The technique of placental perfusion developed for these studies has been reported in detail elsewhere (3). In brief, human placentas, obtained at term from normal pregnancies, are prepared for pulsatile retrograde perfusion with a modified Tyrode's solution to which penicillin and streptomycin are added. The system is maintained at 37° C. The perfusing fluid is exposed to an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide and maintained at pH 7.4. One liter of perfusing fluid is recycled at a rate of about 175 ml. per minute and changed hourly during the eight hours of perfusion. The sterile condition of the perfusate was confirmed by frequent cultures.

Experimental design

Four perfusions were performed. Radioactive estradiol was introduced by adding it with the fresh perfusing fluid at the hours indicated in Table 1. Each time, 1.0 μc. of 4-C^{14}-17β-estradiol in propylene glycol was used. The radioactive estradiol had a specific activity of 4.65 μc. per mg. and was demonstrated to be 99.5 per cent pure on radiochromatography.

To determine the effect of human chorionic gonadotropin (HCG) on the metabolism of the C^{14}-estradiol, HCG was added to the fresh perfusing fluid at the hours indicated in Table 1. A concentration of 20,000 international units of HCG per liter of perfusing fluid was used.

Analyses were performed on about half of the individual hourly perfusates as indicated in Table 1. In addition, the placental tissue from perfusion III was subjected to extraction.

Biochemical procedures

Extraction and identification. Perfusates, and the placental tissue from perfusion III, were stored at −17° C. until the time of extraction. The extraction procedure was as follows: A 250-ml. aliquot of the perfusate was extracted five times with equal volumes of ether. The ether extract was washed with 5 per cent $NaHCO_3$, dried over anhydrous

TABLE 1. DESIGN OF EXPERIMENT

Perfusion No.	Hour							
	1	2	3	4	5	6	7	8
I		C^{14} *		C^{14} *			C^{14} *	
II	HCG	C^{14} HCG *	HCG	C^{14} HCG *	HCG	HCG	C^{14} HCG *	HCG
III		C^{14} *		C^{14} HCG *	HCG	C^{14} HCG *	HCG	C^{14} HCG *
IV		C^{14} *		C^{14} *	HCG	C^{14} HCG *	HCG *	C^{14} HCG *

The perfusion hour during which C^{14}-estradiol (C^{14}) or human chorionic gonadotropin (HCG) were added are marked accordingly. The asterisk (*) indicates perfusion hours for which the perfusates were analyzed. In addition the placenta from perfusion III was subjected to extraction.

sodium sulfate, and evaporated on a rotary evaporator. The residue was distributed in a countercurrent distribution machine, usually after the addition of 100 μg. of carrier estrogen. Upon completion of the distribution, aliquots were taken for fluorometry and measurement of radioactivity. Other aliquots were further processed through countercurrent distribution and paper chromatography, for comparison of the behavior of the radioactive material with that of standard estrogens. Finally, different derivatives such as the methyl ether and the acetylated methyl ether were prepared, and these derivatives were submitted to characterization by countercurrent distribution. All evaporations were carried out at a temperature below 40° C., and either under reduced pressure or under a stream of nitrogen.

The placental tissue from perfusion III was suspended in a 50 per cent saturated solution of ammonium sulfate and extracted with ethanol in a blendor. The ethanol was evaporated and the residue dissolved in water; this mixture was extracted with ether and then processed further in the same manner as the perfusates.

Countercurrent distribution (CCD) was performed using a 100-tube, all-glass, automatic Craig-Post machine. Calculations were made according to Weisiger (4). Solvent systems used were those described by Engel *et al.* (5–7) and Diczfalusy *et al.* (8, 9).

Paper chromatography was carried out by the Bush technique (10) with solvent systems described by Bush (10) and Mitchell and Davies (11). Samples were dried on the paper under a stream of nitrogen. The color of the estrogen standards was developed with ferric chloride-ferricyanide solution (12). Elutions were performed by cutting the appropriate area into small strips and allowing the latter to stand in 25 ml. of methanol overnight, and then extracting twice again with methanol on the following morning.

Fluorometry was accomplished with a Farrand fluorometer developing fluorescence by the method of Diczfalusy (13); 437 mμ was used as the primary, and 487 mμ as the secondary wavelength.

Radioactivity was measured with a D-47 Nuclear Chicago flow counter, both with and without a very thin end-window. In general, planchettes were plated at infinite thinness. When this was not possible, a self-absorption correction curve was constructed and

the results corrected to infinite thinness. Samples were counted to yield a standard error of 2–3 per cent. Chromatograms were scanned by an Atomic thin-window flow counter connected to a rate meter and recorder.

Derivative formation. Methyl ether derivatives were made by the method of Brown (14). Acetylation was performed with pyridine and acetic anhydride at room temperature overnight.

Solvents were purified before use. Benzene and toluene were dried over anhydrous calcium chloride and distilled. Methanol and ethanol were passed through aluminum oxide and distilled. Chloroform, n-hexane, petroleum ether and ligroin were washed with sulfuric acid, dried over anhydrous sodium sulfate and distilled. Carbon tetrachloride was passed through silica gel and then processed like chloroform. Peroxide-free ether was used directly from a freshly opened can.

Estrogen standards. Reference material was graciously supplied as follows: estradiol, estrone and estriol, by the Schering Corporation; 2-methoxyestrone, by Drs. Gallagher and Fishman, Sloan-Kettering Institute; and 16-epiestriol, by Dr. Levitz, New York University.

Generous supplies of HCG ("APL") were given by Ayerst Laboratories through the courtesy of Dr. Jewell.

RESULTS

Radioactivity was extracted from perfusates or placenta and its partition among 17β-estradiol and some of its various metabolites was determined. The metabolites studied included estrone, estriol, 16-epiestriol and 2-methoxyestrone. The evidence for identification of the C^{14}-estrogens is presented and analyzed subsequently. In each instance in which a series of physico-chemical constants was determined, a sequential process was followed; that is, each procedure was performed on material characterized by

TABLE 2. DISTRIBUTION OF RECOVERED ESTROGEN RADIOACTIVITY (%)

Perfusion	Hour	Estradiol	Estrone	Estriol	16-Epiestriol	2-Methoxyestrone
I	2	20.6	79.4	0		
	4	20.7	79.3	0		
	7	15.5	84.5	0		
II	2	15.8	79.7	4.5		0
	4	18.9	74.3	3.8	approx. 1.0	2.0
	7	34.4	64.4	1.2		
III	2	17.1	82.9	0		
	4	21.0	75.2	2.5		1.3
	6	39.6	58.5	1.2		.7
	8	21.6	76.1	0.7		1.6
IV	2	19.5	80.5			
	4	39.1	60.9	0		
	6	29.4	70.6			
	7	27.0	73.0	0		
	8	11.5	88.5	0		
Placenta from perfusion III		34.8	60.5	2.0	1.0	1.7

TABLE 3. EVIDENCE FOR IDENTIFICATION OF C^{14}-ESTRADIOL

Step No.	Procedure*	System	Unknown†	Standard
1	CCD (100T)	70% CH_3OH – 30% H_2O / 40% $CHCl_3$ – 60% CCl_4	K = 1.1	K = 1.1
	Tubes 35–69 ↓			
2	Chromat. (30°C.)	C_6H_6 / 50% CH_3OH – 50% H_2O	R_f = .75	R_f = .75
	↓			
3	Chromat. (23°C.)	Ligroin / CH_3OH	3.8 cm. / 27 hrs.	3.8 cm. / 27 hrs.
	↓			
4	CCD (60T)	40% CH_3OH – 60% H_2O / CCl_4	K = 1.0	K = 1.0
	Tubes 18–42 ↓			
5	CCD of 3-methyl ether (30T)	n-Hexane / 90% CH_3OH – 10% H_2O	K = .28	K = .28

* CCD = countercurrent distribution; T = transfers; Chromat. = paper chromatography.
† K = partition coefficient.

the preceding step. It was thought that more significance could be attached to such evidence than to data from countercurrent distributions or chromatography performed on different aliquots of the same extract.

The extraction procedure used yields only free estrogens. Fifteen to twenty per cent of the perfused radioactivity was recovered in the estrogens identified in the extracts of the perfusates. The distribution of this radioactivity is indicated in Table 2. The radioactivity associated with each estrogen is expressed as a percentage of the total radioactivity identified in the whole extract of the corresponding perfusate.

C^{14}-17β-estradiol

The evidence for the identification of C^{14}-estradiol in the perfusion material is presented in Table 3. The distribution curves of carrier estradiol, determined by fluorometry, were identical with the distribution curves of recovered C^{14}-estradiol determined by radioactivity. No further effort was expended in the identification of the C^{14}-17β-estradiol, because this was the radioactive material originally added.

C^{14}-estrone

The evidence for the identification of C^{14}-estrone from the perfusion material is presented in Table 4. The distribution curves of carrier estrone, determined by fluorometry, were identical with those of the radioactivity of

TABLE 4. EVIDENCE FOR IDENTIFICATION OF C^{14}-ESTRONE

Step No.	Procedure	System	Unknown	Standard
1	CCD (100T)	70% CH_3OH – 30% H_2O / 40% $CHCl_3$ – 60% CCl_4	K = .20	K = .20
	Tubes 4–30			
2	Chromat. (23°C.)	Ligroin / CH_3OH	8.8 cm. / 27 hrs.	8.8 cm. / 27 hrs.
3	CCD (60T)	40% CH_3OH – 60% H_2O / CCl_4	K = .15	K = .15
	Tubes 0–15			
4	Chromat. (30°C.)	C_6H_6 / 50% CH_3OH – 50% H_2O	R_f = .89	R_f = .89
5	CCD (60T)	70% CH_3OH – 30% H_2O / CCl_4	K = 1.2	K = 1.2
	Tubes 21–45			
6	CCD of 3-methyl ether (30T)	Petroleum ether / 90% CH_3OH – 10% H_2O	K = .87	K = .87

recovered C^{14}-estrone. Inasmuch as the conversion of estradiol to estrone by placental tissue has been well documented (15, 16), the evidence for the presence of C^{14}-estrone in the extracts was considered satisfactory. No correlation was evident between the conversion of estradiol to estrone and the addition of HCG.

C^{14}-estriol

The evidence for the identification of C^{14}-estriol is presented in Table 5. Fluorometry curves of carrier estriol coincided with distribution curves of the radioactivity of C^{14}-estriol in steps 1 and 3. The distribution curves of radioactivity in steps 4 and 5 coincided with the theoretical curves. An example of this is shown in Figure 1, which shows the distribution curve for step 5, Table 5.

Insufficient material was available for further studies. However, partition coefficients identical with those of standard material were obtained with both the 3-methyl-ether and then the diacetate of the 3-methyl-ether of C^{14}-estriol, in addition to the other indicated partition coefficients and chromatographic mobility. The evidence appears adequate to justify the statement that a partial conversion of C^{14}-estradiol to C^{14}-estriol took place during the perfusion.

From Tables 1 and 2 it may be seen that C^{14}-estriol was found only after HCG had been added to the perfusing fluid. Thus it was not found in per-

TABLE 5. EVIDENCE FOR IDENTIFICATION OF C^{14}-ESTRIOL

Step No.	Procedure	System	Unknown	Standard
1	CCD 60T)	70% CH_3OH – 30% H_2O / 40% $CHCl_3$ – 60% CCl_4	K = 8.2	K = 8.2
	Tubes 48–60 ↓			
2	Chromat. (30°C.)	C_6H_6 / 50% CH_3OH – 50% H_2O	11.5 cm. / 24.5 hrs.	11.5 cm. / 24.5 hrs.
	↓			
3	CCD (30T)	70% CH_3OH – 30% H_2O / 80% $CHCl_3$ – 20% CCl_4	K = 1.0	K = 1.0
	Tubes 10–24 ↓			
4	CCD of 3-methyl ether (29T)	40% CH_3OH – 60% H_2O / CCl_4	K = 0.84	K = 0.85
	Tubes 6–24 ↓			
5	CCD of 3-methyl ether, 16, 17 diacetate (21T)	n-Hexane / 90% CH_3OH – 10% H_2O	K = 0.92	K = 0.96

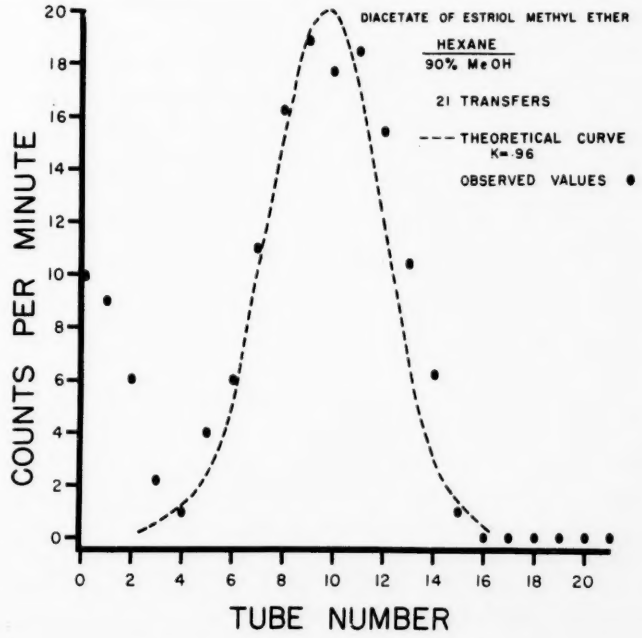

FIG. 1. Countercurrent distribution, step 5, Table 5, showing relationship of distribution of radioactivity of unknown to theoretical distribution curve of diacetate of estriol-3-methyl ether. The radioactivity in tubes 0, 1 and 2 was not identified.

TABLE 6. EVIDENCE FOR IDENTIFICATION OF C^{14}-16-EPIESTRIOL

Step No.	Procedure	System	Unknown	Standard
1	CCD (100T)	$\frac{70\%\ CH_3OH - 30\%\ H_2O}{40\%\ CHCl_3 - 60\%\ CCl_4}$		
	Tubes 53–100 ↓			
2	CCD (40T)	$\frac{50\%\ CH_3COOC_2H_5 - 50\%\ C_6H_{12}}{50\%\ C_2H_5OH - 50\%\ H_2O}$		
	Tubes 6–21 ↓			
3	CCD (30T)	$\frac{C_6H_6}{H_2O}$		K = 3.4
	Tubes 10–30 ↓			
4	CCD (60T)	$\frac{C_6H_6}{H_2O}$		K = 3.4
	Tubes 40–60 ↓			
5	CCD (30T)	$\frac{40\%\ CH_3COOC_2H_5 - 60\%\ C_6H_{12}}{40\%\ C_2H_5OH - 60\%\ H_2O}$	K = 1.7	K = 1.6
	Tubes 14–25 ↓			
6	*CCD of 3-methyl* ether (20T)	$\frac{n\text{-Hexane}}{80\%\ CH_3OH}$	K = 1.5	K = 1.4

fusion I or at the beginning of perfusion II; it was present in the latter part of perfusion II and throughout perfusion III. However, in perfusion IV no C^{14}-estriol was found despite the addition of HCG in the last four hours.

The possibility was considered that a labeled contaminant might be mistaken for C^{14}-estriol. However, the contaminant would have to possess partition coefficients similar to those of estriol in each of the several distribution systems used, as well as partition coefficients similar to those of estriol methyl ether and the diacetate of estriol methyl ether. The contaminant would also have to be absent when HCG is not added. It would be unlikely for contaminating C^{14}-estradiol or C^{14}-estrone to come through the separation procedures, and an unlikely coincidence for such a contaminant to appear only in the experiments with added HCG.

C^{14}-16-epiestriol

The evidence for the identification of C^{14}-16-epiestriol in perfusion material is presented in Table 6. Partition coefficients for the early steps were not obtained, either because separation from other C^{14} estrogens (estradiol and estriol) was inadequate or because no counting of radioactivity was performed before the next step. Therefore the band of tubes which by calculation from the theoretical partition coefficient contained 16-epiestriol, was used for the subsequent distribution. In steps 5 and 6 good agreement

of distribution curves and partition coefficients with the standard values was obtained.

The evidence for the identity of C^{14}-16-epiestriol cannot be considered conclusive and permits only a tentative identification. However, the agreement of the partition coefficients of the compound and its 3-methyl ether with the corresponding coefficients of standard material following the several indicated isolation steps suggests strongly that the isolated radioactivity was identical with 16-epiestriol.

In addition to the foregoing material which was found in the placental tissue from perfusion III, radioactivity was isolated from the perfusate for hour 4 of perfusion II, which after several isolation steps and methylation behaved like the 3-methyl ether of 16-epiestriol on countercurrent distribution.

C^{14}-2-methoxyestrone

The evidence for the identification of C^{14}-2-methoxyestrone in the perfusion material is presented in Table 7. Following a preliminary isolation step, three sequential procedures showed, first, chromatographic mobility equal to that of standard material, and then close correspondence of two distribution coefficients with standard values (Fig. 2). Although this cannot be considered conclusive evidence, it appears reasonable to base a tentative identification on these constants.

Although the search was not as extensive as that for C^{14}-estriol, there

TABLE 7. EVIDENCE FOR IDENTIFICATION OF C^{14}-2 METHOXYESTRONE

Step No.	Procedure	System	Unknown	Standard
1	CCD (60T) Tubes 0–13	70% CH_3OH – 30% H_2O / 40% $CHCl_3$ – 60% CCl_4		
2	Chromat (23°C.)	Ligroin / CH_3OH	16.7 cm. / 26 hrs.	16.7 cm. / 27 hrs.
3	CCD (60T) Tubes 2–20	70% CH_3OH – 30% H_2O / CCl_4	K = .25	K = .24
4	CCD (29T)	80% CH_3OH – 20% H_2O / CCl_4	K = .66	K = .74*

* This partition coefficient was determined by Engel (5). Reference material was not available to re-determine the value of K at the time the unknown was subjected to CCD.

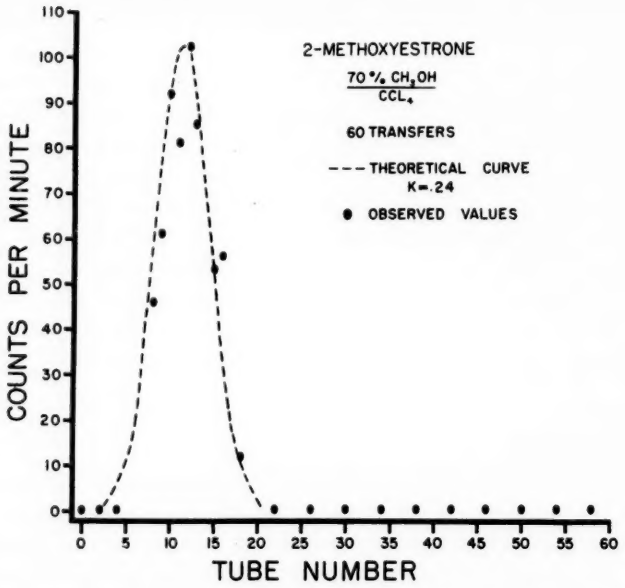

FIG. 2. Countercurrent distribution, step 3, Table 7, showing relationship of distribution of radioactivity of unknown to theoretical distribution of 2-methoxyestrone.

was a suggestion of a correlation with HCG. No C^{14}-2-methoxyestrone was found in the perfusate for hour 2 of perfusion II. The amounts indicated were found for hour 4 of perfusion II and for several other perfusion hours, as well as in the placental tissue from perfusion III.

DISCUSSION

Although estriol is the major estrogen of human pregnancy urine (17), its origin during pregnancy is not clear. It has been suggested that estriol may be predominantly a direct secretory product of the placenta (18) rather than a product of the conversion of estrone-estradiol in placental or extra-placental sites. Evidence for this comes from the failure of previous attempts to show conversion of estradiol to estriol by placental tissue homogenates (15) or perfused cotyledons (16); from the increasing ratio of estriol to estrone-estradiol excreted in urine as pregnancy progresses (19); from the presence of large amounts of free estriol in the term placenta (13); and from the demonstration of aromatization of Δ^5-androstenetriol to estriol by placental preparations (20). However, the ability of maternal (21) and fetal (22) organisms to convert estradiol to estriol has been shown. A possible site of conversion seems to be the fetal liver; it has been shown that

liver slices from human fetuses convert estradiol to estriol with a 3.8 per cent yield (23).

The demonstration of the ability of the perfused placenta *in vitro* to convert C^{14}-estradiol to C^{14}-estriol adds another possible pathway to those indicated. Of perhaps even greater significance is the fact that this conversion was found only after addition of HCG to the perfusing fluid. The effect of variables other than HCG may be indicated in one of the perfusion studies where no C^{14}-estriol was found despite the addition of HCG. Large and rather constant amounts of HCG are produced by the placenta during the second and third trimesters of pregnancy (13). The role of HCG during this period is not known. Our previous study of the effect of HCG on the estrogen stimulation of citrate metabolism by the perfused placenta (3) suggested that a possible role for HCG might be sought in placental autoregulatory processes. Now the additional finding of an apparent relationship between HCG and the conversion of estradiol to estriol again supports the assumption of self-regulatory processes in placental function.

The pathway for conversion of 17β-estradiol to estriol may be by con-

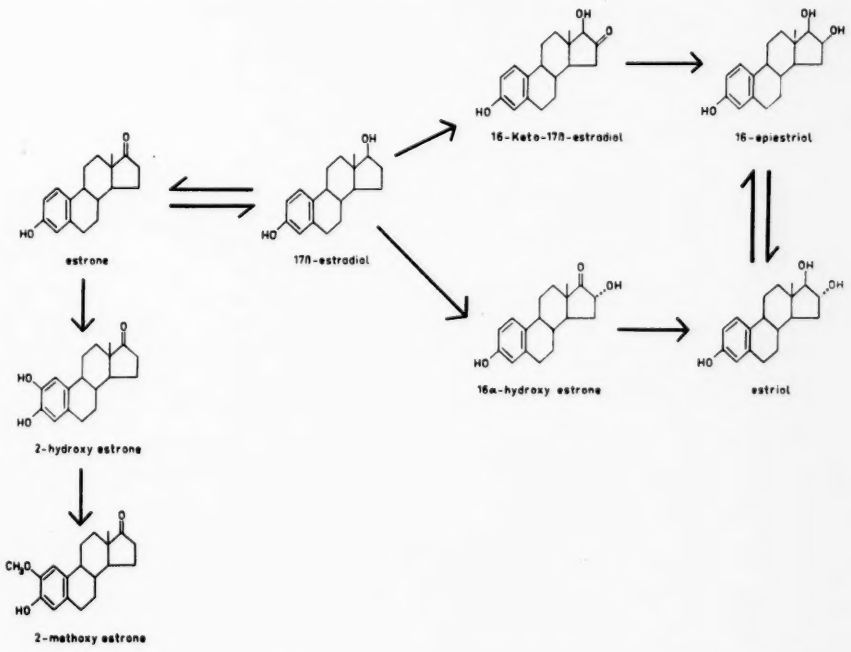

FIG. 3. Tentative scheme to illustrate pathways for the metabolism of 17β-estradiol observed in perfused placentas.

version of 17β-estradiol to 16-keto-17β-estradiol and then to estriol. This has been shown *in vivo* (24); however, placental 10,000$\times$g supernatant does not convert 16-keto-17β-estradiol to estriol (25). A second pathway may proceed via conversion of 17β-estradiol to 16α-hydroxyestrone, which has been isolated from pregnancy urine extracts (26), and then to estriol, as has been shown *in vivo* (27) as well as in human placental preparations (28). A third possibility, namely, direct 16-hydroxylation of 17β-estradiol has not been demonstrated. Figure 3 presents a tentative scheme for the metabolism of 17β-estradiol in perfused placentas.

The tentative identification of C^{14}-16-epiestriol following perfusion of C^{14}-17β-estradiol was not unexpected in view of the demonstrated conversion *in vivo* of estriol to 16-keto-17β-estradiol and 16-epiestriol (29), as well as the presence of 16-epiestriol in placental tissue (9). 2-Methoxyestrone, also tentatively identified in these perfusion studies, has been isolated from the urine of both nonpregnant (30) and pregnant (31) subjects but has not previously been found in placental tissue. The pathway for conversion of 17β-estradiol to 2-methoxyestrone appears to be a two-step mechanism involving 2-hydroxyestradiol and its subsequent methylation (32). Although the experimental conditions under which C^{14}-16-epiestriol and C^{14}-2-methoxyestrone were found seem to suggest that added HCG might be needed for these conversions, the relatively limited number of experiments do not allow a definite conclusion.

These perfusion studies also showed the conversion of C^{14}-17β-estradiol to C^{14}-estrone. This conversion has been previously demonstrated by others in placental tissue slices (15) and perfused cotyledons (16), and the enzyme involved in this conversion has been partially purified and characterized (33).

The demonstration of pathways of estrogen metabolism by means of studies on the perfused placenta suggests many further investigations. Included would be analysis of the biosynthesis and metabolism of various steroids and the possible role of HCG in the regulation of these processes. Extension of these studies to placentas obtained earlier than term might allow for even more active preparations. The recent demonstration of the feasibility of *in vivo* perfusion of human placentas also indicates a method for further study (34).

Acknowledgment

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17α-HYDROXYPROGESTERONE—A SALT-LOSING STEROID: RELATION TO CONGENITAL ADRENAL HYPERPLASIA

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ABSTRACT

Sodium, chloride and potassium metabolic balance studies were performed on 3 "normal" subjects with intact adrenal function and 2 patients with adrenocortical insufficiency treated with desoxycorticosterone acetate (DCA) and cortisone acetate. During the period of daily oral administration of 500 mg. of nonesterified 17α-hydroxyprogesterone, there resulted an excessive loss of salt, significantly above control values in all "normal" subjects as well as in both patients with adrenal insufficiency receiving DCA concurrently. Changes in potassium excretion were variable. These changes in electrolytes promptly disappeared upon withdrawal of 17α-hydroxyprogesterone. No salt loss was produced during a four-day course of 17α-hydroxyprogesterone in 2 patients with adrenal insufficiency receiving no salt-retainer. The saluretic activity of 17α-hydroxyprogesterone appears to be dependent upon the presence of an adrenal salt-retaining steroid. Its mechanism of action is probably based on inhibition of the renal tubular effects of desoxycorticosterone and aldosterone. These observations are offered as an explanation for the active salt loss which often occurs in congenital adrenal hyperplasia, emphasizing the possible role of 17α-hydroxyprogesterone as the adrenal principle involved.

DEMONSTRABLE influences of adrenocortical steroids on salt and water metabolism in the human have until recently been limited to their sodium-retaining properties. Observations that indicate modification of this quality under certain abnormal or experimental circumstances have led to speculation concerning the elaboration by the adrenal cortex of a hormone endowed with the ability to promote salt loss.

In congenital adrenal hyperplasia with virilism, conspicuous salt loss in association with addisonian-like symptoms occurs in about one-third of the patients (1). Whereas administration of adrenocorticotropic hormone (ACTH) exaggerates the salt loss in these patients (1–5), administration of

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glucocorticoids is corrective (6). Endogenous aldosterone, even in elevated amounts, is apparently unable to prevent the salt loss in these children. These observations suggest that the adrenal cortex, under the control of the anterior pituitary, may secrete a substance which either chemically modifies the sodium-retaining action of aldosterone[1] or exerts a direct influence on renal tubular mechanisms to antagonize reabsorption of sodium.

In congenital adrenal hyperplasia, the impairment in 21-carbon hydroxylation retards the biosynthesis of hydrocortisone (7). The resultant excessive secretion of ACTH (8) inappropriately accelerates the production of hydrocortisone precursors. In this manner, 17α-hydroxyprogesterone levels rise in the blood, and the metabolic products of 17α-hydroxyprogesterone—chiefly pregnanetriol, 17α-hydroxypregnanolone, and 11-ketopregnanetriol—are excreted in excess in the urine (1, 9–11). The metabolic product of progesterone—pregnanediol—is usually not elevated in the urine (12), indicating the ability of the 17α-hydroxylation enzyme system to accommodate to the increased demand in most instances.

Because of the accumulation of 17α-hydroxyprogesterone in congenital adrenal hyperplasia, the demonstration of a salt-losing effect in response to ACTH in this syndrome, and the observation that the administration of either ACTH (2, 4, 7, 13, 14) or 17α-hydroxyprogesterone (3, 7, 15) can provoke a further rise in pregnanetriol and neutral 17-ketosteroid excretion, it appears logical to examine 17α-hydroxyprogesterone for possession of a salt-losing potential. Its recognizable action would depend on its presence in the circulation in excessive amounts. Accordingly, relatively large amounts of 17α-hydroxyprogesterone were administered to 3 subjects with normal adrenal function, as well as to 2 bilaterally adrenalectomized, oophorectomized patients to observe any influence of the steroid on sodium, chloride and potassium balance.

METHODS

Five patients in The Mount Sinai Hospital were studied. These included 3 "normal" adult male subjects with no evidence of fluid retention and with intact hepatic, renal and

[1] Trivial names are used in the text for the following compounds: Aldosterone is 11β,21-dihydroxy-18-oxo-4-pregnene-3,20-dione. Hydrocortisone is 11β,17α,21-trihydroxy-4-pregnene-3,20-dione. 11-Desoxyhydrocortisone (compound S) is 17α,21-dihydroxy-4-pregnene-3,20-dione. Corticosterone is 11β,21-dihydroxy-4-pregnene-3,20-dione. Desoxycorticosterone (DOC) is 21-hydroxy-4-pregnene-3,20-dione. Pregnenolone is 3β-hydroxy-5-pregnene-20-one. Progesterone is 4-pregnene-3,20-dione. 17α-Hydroxyprogesterone is 17α-hydroxy-4-pregnene-3,20-dione. Pregnanetriol is pregnane-3α,17α,20α-triol. 17α-Hydroxypregnanolone is 3α,17α-dihydroxy-pregnane-20-one. 11-Ketopregnanetriol is 3α,17α,20α-trihydroxypregnane-11-one. Pregnanediol is pregnane-3α,20α-diol. Cortisone is 17α,21-dihydroxy-4-pregnene-3,11,20-trione. Prednisone is 17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione.

TABLE 1. BALANCE STUDY IN A "NORMAL" MALE (D.K.)

Balance period*	Medication	Urinary sodium (mEq./24 hrs.)	Urinary chloride (mEq./24 hrs.)	Urinary potassium (mEq./24 hrs.)	Urinary pregnanetriol (mg./24 hrs.)	Total urine volume (ml./24 hrs.)	Serum sodium (mEq./L.)	Serum chloride (mEq./L.)	Serum potassium (mEq./L.)
1		67.6	58.4	59.6		1005	141	99	5.0
2		67.2	66.6	55.7	0.6	1042			
3		70.4	65.6	60.3	0.6	1220	143	97	5.4
4†	17-OH-	36.4	48.8	48.5	6.4	995			
5	proges-	86.8	74.2	70.4	5.8	1022			
6	terone,	96.1	81.8	53.3	8.6	1145	137	98	5.1
7	500 mg.	115.0	94.1	55.9	11.3	1232			
8	daily	146.7	129.4	63.6	16.4	1570	138	96	4.9
9		88.4	93.8	53.4	2.8	1238			
10		76.8	78.2	71.4	1.3	1392			
11		77.1	87.5	64.9		1090	140	99	4.7
12		78.2	80.0	68.7		990	143	99	5.4

* Each balance period represents an interval of two days. All urinary and serum values are the average for each two-day balance period. Electrolyte intakes are indicated in Figure 1.
† Diarrhea occurred during fourth balance period.

endocrine function, and 2 adult female subjects following bilateral adrenalectomy and oophorectomy for carcinoma of the breast with metastases. In the 2 patients with adrenal insufficiency there was also no demonstrable fluid retention or abnormality involving the liver, kidneys or pituitary gland.

For all subjects, the intake of sodium, chloride and potassium was constant throughout the study. Water intake was not restricted. A six- to nine-day period of equilibration to the balance diet was permitted before exposure to the drug under study.

The daily 24-hour urine output and serum levels of sodium and potassium were measured by flame photometry, with an internal standard. Urinary and serum chloride levels were determined by the method of Schales and Schales (16), and urinary and serum creatinine by the alkaline picrate method (17). These values were averaged over two-day periods. The 24-hour urinary excretion of pregnanetriol was determined by the method of Bongiovanni and associates (18, 19) before, during, and after administration of 17α-hydroxyprogesterone in order to substantiate adequate absorption and utilization of the compound.

17α-Hydroxyprogesterone in gelatin capsules was administered orally in the nonesterified form[2] in a dosage of 500 mg. daily. Desoxycorticosterone, when administered to the adrenalectomized patients, was injected intramuscularly as the acetate (DCA) in a dosage of 5 mg. daily. These 2 patients were maintained with cortisone acetate (37.5 mg. daily by mouth) throughout the period of observation, having received a similar dosage for at least ten days prior to study. In the adrenalectomized patient (I.S.) receiving mineralocorticoid therapy, the last previous injection of 50 mg. of desoxycorticosterone trimethylacetate had been given four weeks earlier.

RESULTS

"Normal" subjects

Results were similar in all the "normal" male subjects (Tables 1–3 and Figs. 1–3). During the control period, sodium, chloride and potassium equilibrium or near-balance was achieved within six to nine days after in-

[2] The 17α-hydroxyprogesterone (nonesterified) was generously supplied by Dr. Harry W. Rudel of Syntex Chemical Corporation, New York, N.Y.

TABLE 2. BALANCE STUDY IN A "NORMAL" MALE (J.T.)

Balance period*	Medication	Urinary sodium (mEq./24 hrs.)	Urinary chloride (mEq./24 hrs.)	Urinary potassium (mEq./24 hrs.)	Urinary pregnanetriol (mg./24 hrs.)	Total urine volume (ml./24 hrs.)	Serum sodium (mEq./L.)	Serum chloride (mEq./L.)	Serum potassium (mEq./L.)
1		13.4	16.9	65.6		1085			
2		12.5	15.0	59.8	0.02	1035	144	99	4.7
3		9.2	18.8	74.2	0.2	1125	142	101	4.7
4	17-OH	12.6	15.8	62.0	4.7	1030			
5	proges-	14.0	13.0	62.1		1258			
6	terone,	19.8	9.5	67.8	9.2	1425	139	100	4.8
7†	500 mg.	4.5	8.0	70.2	5.6	1158			
8	daily	13.8	6.9	63.0		1000	145	102	4.8
9		22.9	9.1	57.8	18.5	958			
10		29.0	13.5	54.9		1115	140	103	4.7
11		21.8	17.6	68.8	18.6	1222			
12		26.4	62.8	63.6		895			
13		28.7	61.6	56.1	12.8	950	138	99	4.7
14		8.0	12.4	53.4	0.4	768	137	102	4.6
15		6.7	16.4	55.5	0.3	1215	141	103	4.5
16		9.7	18.1	63.2		1070			

* Each balance period represents an interval of two days. All urinary and serum values, except pregnanetriol, are the average for each two-day balance period. Pregnanetriol values represent a single 24-hour urine. Electrolyte intakes are indicated in Figure 2.
† A muscle biopsy was performed on the thirteenth day of the balance study.

stitution of a constant diet. A dose of 500 mg. of 17α-hydroxyprogesterone was then administered by mouth daily for ten to twenty days. Urinary sodium and chloride excretion began to rise within six days, attaining a peak after six to nine days. Except for a brief period of diarrhea in one subject, during which there was a fall in urinary electrolyte concentrations (Fig. 1), and following a muscle biopsy in another subject, when there was transient sodium and chloride retention (Fig. 2), negative sodium balance persisted throughout the period of drug administration. Within seventy-

TABLE 3. BALANCE STUDY IN A "NORMAL" MALE (S.A.)

Balance period*	Medication	Urinary sodium (mEq./24 hrs.)	Urinary chloride (mEq./24 hrs.)	Urinary potassium (mEq./24 hrs.)	Urinary pregnanetriol (mg./24 hrs.)	Total urine volume (ml./24 hrs.)	Serum sodium (mEq./L.)	Serum chloride (mEq./L.)	Serum potassium (mEq./L.)
1		15.3	18.8	61.0		1480			
2		14.2	18.0	69.4	0.5	1265	144	105	4.5
3		12.4	16.4	58.8	0.4	985			
4	17-OH-	30.8	16.3	49.2		712			
5	proges-	27.6	16.0	50.2	4.5	1010	142	103	4.6
6	terone,	35.2	22.2	60.9		1052	140	103	4.6
7	500 mg.	34.8	22.0	59.5	3.2	1225			
8	daily	37.2	22.8	56.5		1040	136	103	4.4
9		29.7	18.6	51.5	0.5	878			
10		20.7	19.5	51.4	0.1	1142	136	106	4.6
11		16.6	16.0	53.8	0.04	968			
12		6.7	10.0	50.9	0.04	1358	139	103	4.5
13	17-OH-	21.7	28.0	45.6	0.2	1065			
14	proges-	19.8	18.5	50.9	0.7	1108	137	100	5.1
15	terone,	26.6	17.3	65.2	11.4	1400			
16	500 mg.	26.4	18.0	73.9		1578	135	100	4.6
17	daily	24.0	17.5	76.9		1205			

* Each balance period represents an interval of two days. All urinary and serum values, except pregnanetriol, are the average for each two-day balance period. Pregnanetriol values represent a single 24-hour urine. Electrolyte intakes are indicated in Figure 3.

two hours after the withdrawal of 17α-hydroxyprogesterone, urinary sodium and chloride levels fell, ultimately approaching equilibrium with the dietary intake of these electrolytes. In the one subject to whom a second ten-day course of 17α-hydroxyprogesterone was given, negative sodium balance was again promptly achieved and sustained during the

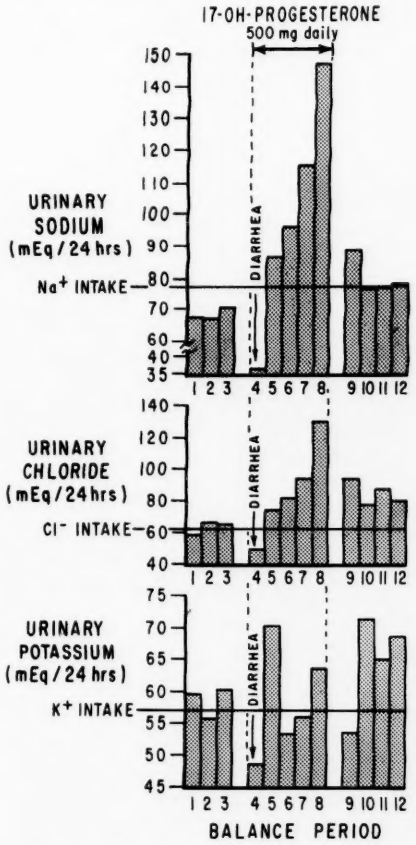

FIG. 1. Effects of 17α-hydroxyprogesterone on electrolyte balance in a "normal" male (D.K.). Here and in Figure 2, each balance period represents an interval of two days. Urinary and serum electrolyte values are the average for each two-day balance period. Sodium, chloride and potassium intakes were 77.8, 62.6, and 57.0 mEq. per day, respectively. Diarrhea occurred during the fourth balance period.

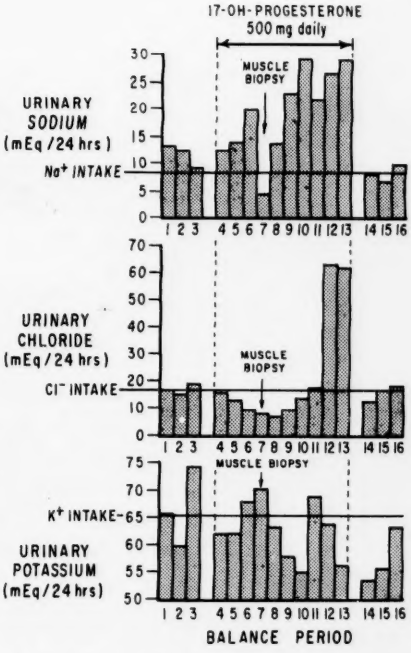

FIG. 2. Effects of 17α-hydroxyprogesterone on electrolyte balance in a "normal" male (J.T.). Sodium, chloride, and potassium intakes were 8.4, 16.6, and 65.4 mEq. per day, respectively. A muscle biopsy was performed on the thirteenth day of the balance study.

period of administration, though at a somewhat lower level of sodium excretion than that attained in the initial trial (Fig. 3).

Adrenalectomized, oophorectomized patients

Both patients were maintained throughout the study by oral administration of 37.5 mg. of cortisone daily, a dosage which they had received for

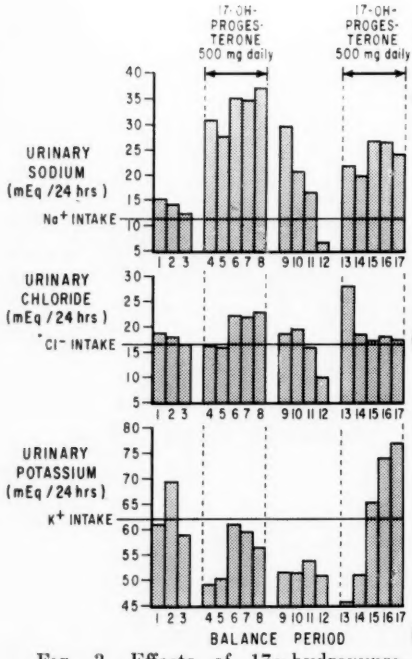

FIG. 3. Effects of 17α-hydroxyprogesterone on electrolyte balance in a "normal" male (S.A.). Here and in Figure 4, each balance period represents an interval of two days. Urinary and serum electrolyte values are the average for each two-day balance period. Sodium, chloride, and potassium intakes were 11.4, 16.6, and 62.2 mEq. per day, respectively.

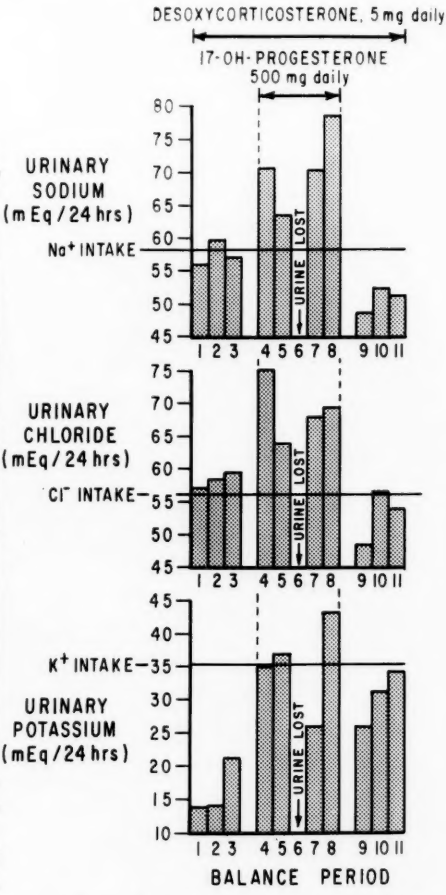

FIG. 4. Effects of 17α-hydroxyprogesterone on electrolyte balance in a bilaterally adrenalectomized, oophorectomized female treated with desoxycorticosterone (M.C.). Sodium, chloride, and potassium intakes were 58.4, 56.3, and 35.4 mEq. per day, respectively.

at least ten days prior to the investigation. In Patient M.C., who received 5 mg. of DCA per day intramuscularly from the outset, there was a slightly positive sodium balance during the control period of six days. Upon the daily oral administration of 500 mg. of 17α-hydroxyprogesterone for ten days, there was a prompt loss of sodium and chloride in the urine, significantly exceeding the dietary intake. Following withdrawal of 17α-hydroxyprogesterone, a positive sodium and chloride balance was rapidly restored as treatment with DCA was continued (Table 4 and Fig. 4).

The other adrenalectomized patient (I.S.) demonstrated equilibrium between urinary and dietary sodium and chloride during the four-day control period. Administration of 500 mg. of 17α-hydroxyprogesterone daily for

TABLE 4. BALANCE STUDY IN A BILATERALLY ADRENALECTOMIZED FEMALE (M.C.)

Balance period*	Medication†	Urinary sodium (mEq./24 hrs.)	Urinary chloride (mEq./24 hrs.)	Urinary potassium (mEq./24 hrs.)	Urinary pregnanetriol (mg./24 hrs.)	Total urine volume (ml./24 hrs.)	Serum sodium (mEq./L.)	Serum chloride (mEq./L.)	Serum potassium (mEq./L.)
1		56.0	57.2	13.8		708	143	98	3.4
2		59.7	58.5	14.0	0.6	898			
3		57.1	59.5	21.2	0.1	1195	144	99	3.6
4	17-OH-progesterone, 500 mg. daily	70.6	75.0	35.0	13.9	1100	140	97	3.5
5		63.5	63.9	37.0		805	142	96	3.7
6		—	urine	lost					
7		70.3	67.9	26.0	59.8	905			
8		78.5	69.4	43.2		890	139	97	3.6
9		48.7	48.5	25.8		705			
10		52.4	56.6	31.2	4.8	1052	140	98	3.6
11		51.3	54.0	34.3	0.2	1002	142	97	3.6

* Each balance period represents an interval of two days. All urinary and serum values, except pregnanetriol, are the average for each two-day balance period. Pregnanetriol values represent a single 24-hour urine. Electrolyte intakes are indicated in Figure 4.

† Desoxycorticosterone acetate (5 mg.) and cortisone (37.5 mg.) were administered daily throughout the balance study.

four days (prior to the use of DCA) did not alter the sodium, chloride or potassium concentrations in the urine. Injections of DCA were then begun, resulting in rapid retention of salt. The development of weakness and mild hypotension necessitated additional administration of glucocorticoids, so prednisone was started in a dosage of 15 mg. daily. This resulted in disappearance of the salt retention, and the development of a slightly negative sodium and chloride balance. Following twelve days of prednisone therapy, this hormone was discontinued; there was a progressive decline in the urinary sodium level, with a more delayed fall in the chloride level. When the degree of sodium retention was similar to that seen before prednisone was given, a six-day course of 17α-hydroxyprogesterone was begun in a dosage of 500 mg. daily. On this occasion, a progressive increase in urinary sodium and chloride concentrations ensued within two days. The effects were reversed by the subsequent withdrawal of 17α-hydroxyprogesterone from the regimen (Table 5 and Fig. 5).

Another patient was studied briefly—a man with long-standing Addi-

TABLE 5. BALANCE STUDY IN A BILATERALLY ADRENALECTOMIZED FEMALE (I.S.)

Balance period*	Medication†	Urinary sodium (mEq./ 24 hrs.)	Urinary chloride (mEq./ 24 hrs.)	Urinary potassium (mEq./ 24 hrs.)	Urinary pregnanetriol (mg./ 24 hrs.)	Total urine volume (ml./ 24 hrs.)	Serum sodium (mEq./ L.)	Serum chloride (mEq./ L.)	Serum potassium (mEq./ L.)
1		168.6	135.1	97.7	0.9	2395			
2		168.5	127.2	96.5	0.6	2215	139	98	5.3
3	17-OH-progesterone,	167.7	115.7	92.8	29.1	1725			
4	500 mg. daily	166.8	119.1	97.6	28.8	1795	140	99	5.4
5	DCA, 5 mg.	107.4	84.2	86.0	6.1	1458	141	98	5.6
6	daily	70.9	55.6	98.0	2.1	2158	145	103	5.6
7	DCA, 5 mg.	82.2	100.0	96.7	0.2	2098			
8	daily	148.0	135.8	108.5		2252			
9	plus	192.0	168.1	100.5		1962	143	101	5.0
10		175.8	164.0	97.8		1785			
11	prednisone,	163.7	147.6	91.0		1845			
12	15 mg. daily	177.5	179.6	98.5		1980			
13	DCA, 5 mg.	179.2	170.8	93.2		1615	137	99	4.4
14	daily	165.3	168.2	80.2		1620			
15		138.4	182.8	96.4		1780	140	98	4.2
16		142.3	171.6	105.4		2005			
17	↓	82.5	112.9	105.0	0.7	1750	144	97	4.2
18	17-OH-progesterone,	87.1	114.6	103.4	19.6	1500	148	100	4.4
19	500 mg. plus	145.0	138.2	90.6	32.8	1440			
20	DCA, 5 mg. daily	198.2	153.9	79.4	47.4	1432	145	96	4.6
21	DCA, 5 mg.	163.5	179.2	85.5	19.5	1540			
22	daily	136.3	144.8	87.6	2.4	1500	148	100	4.2

* Each balance period represents an interval of two days. All urinary and serum values are the average for each two-day balance period. Electrolyte intakes are indicated in Figure 5.

† Cortisone (37.5 mg.) was administered daily throughout the balance study.

son's disease, who received 50 mg. of cortisone daily and a constant intake of sodium, chloride and potassium in his diet, which contained 145.2 mEq. of sodium per day. Sodium and chloride balance were moderately positive. The administration of 500 mg. of 17α-hydroxyprogesterone daily for four days had no significant influence on the urinary excretion of sodium, chloride or potassium. The study was discontinued because of the development of hypotension.

In all subjects studied, fluctuations in urinary chloride levels tended to parallel those in urinary sodium. Alterations in urinary potassium concentration were inconsistent, but usually varied inversely with those of sodium and chloride. Serum electrolyte levels generally reflected the changes noted in urinary excretion (Tables 1–5). The glomerular filtration rate, as estimated by the endogenous creatinine clearance, did not change significantly as a result of 17α-hydroxyprogesterone administration. Urinary pregnanetriol excretion was normal during the control periods, but became markedly elevated during treatment with 17α-hydroxyprogesterone. This elevation was from two and a half to five times greater in the subjects with adrenal insufficiency than in those with intact adrenal function.

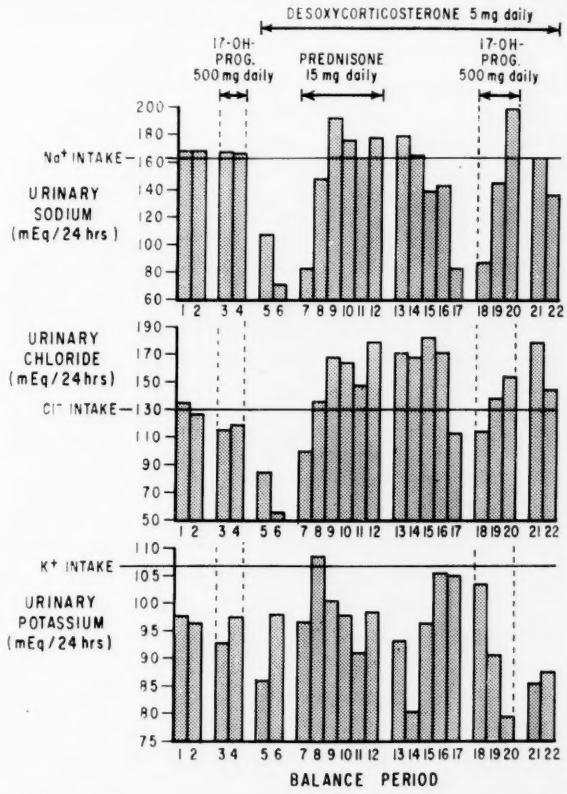

FIG. 5. Effects of 17α-hydroxyprogesterone on electrolyte balance in a bilaterally adrenalectomized, oophorectomized female before and during administration of desoxycorticosterone (I.S.). Each balance period represents an interval of two days. Urinary and serum electrolyte values are the average for each two-day balance period. Sodium, chloride, and potassium intakes were 163.3, 130.5, and 106.8 mEq. per day, respectively.

DISCUSSION

In the nonhypertensive form of congenital adrenal hyperplasia, the excretion of metabolites of hydrocortisone precursors in salt-losers appears to exceed that in patients of comparable age who are not salt-losers (2, 10, 20–24). This suggestion, together with observations by others (22, 25) that hydrocortisone is undetectable or exceedingly low in the salt-losers, lends further support to the concept that interference with 21-hydroxylation of adrenal steroids is more complete in patients who are unable to retain salt adequately. That this block is not total is evidenced by the ability of these

patients to manufacture, at least in most instances, a normal or slightly elevated complement of aldosterone (1, 21, 26). Nevertheless, these patients are apparently unable to compensate fully for their salt and water deficit, since aldosterone excretion, even under the additional stimulus of salt restriction, can at best rise only slightly (27).

The less pronounced 21-hydroxylase activity in salt-losers implies a greater accumulation of 17α-hydroxyprogesterone in the circulation. Since dosages up to 250 mg. daily of 17α-hydroxyprogesterone have failed to evoke a natriuresis (28–30), a larger dosage was selected for the present studies. This dosage in "normal" subjects maintaining a low sodium intake, produced urinary levels of pregnanetriol comparable to those seen in untreated patients with the salt-losing form of congenital adrenal hyperplasia. Under these circumstances, significant natriuresis and chloruresis were elicited, indicating the ability of 17α-hydroxyprogesterone to antagonize the salt-retaining properties of aldosterone and desoxycorticosterone (DOC). This was corroborated by the results in patients with DOC-treated adrenal insufficiency, who exhibited salt loss upon administration of 17α-hydroxyprogesterone only when DOC was present. It is unlikely that any salt-losing action of 17α-hydroxyprogesterone in congenital adrenal hyperplasia is due to interference with the secretion or excretion of aldosterone or desoxycorticosterone, since aldosterone excretory rates are not impaired in patients with the salt-losing syndrome.

Since the effect of the urinary metabolites of 17α-hydroxyprogesterone has not been studied adequately in humans, it is uncertain whether one of these metabolites or the parent compound is the steroid that directly accounts for salt loss. Pregnanetriol excretion in patients with adrenal insufficiency receiving 17α-hydroxyprogesterone is considerably greater than in subjects with intact adrenals, but is not accompanied by any greater loss of salt. This suggests that pregnanetriol is probably not the active salt-losing compound.

The previous search for an adrenal salt-losing steroid in congenital adrenal hyperplasia has focused on the chromatographic separation of steroids from the urine of patients exhibiting active salt loss. This method has yielded certain fractions capable of inducing loss of sodium in adrenalectomized male rats. Neher and his associates (31, 32) isolated 3β,16α-dihydroxy-allopregnan-20-one and 3α,16α-dihydroxy-pregnan-20-one from the urine of patients with congenital adrenal hyperplasia and a salt-losing syndrome, the former compound also having been identified in hog adrenals. These steroids produced natriuresis in adrenalectomized rats, but when the former compound was administered to humans by Coppage and Liddle (33), no change in sodium balance was observed with the doses tested. Klein *et al.* (30) noted loss of salt in adrenalectomized rats following

injections of an eluate of an unknown paper chromatographic fraction separated from the urine of infants with congenital adrenal hyperplasia, as well as newborn premature infants without adrenal disease. Recently Rosemberg *et al.* (34) separated an impure chromatographic fraction from the urine of patients with the salt-losing form of congenital adrenal hyperplasia. This was thought to contain predominantly 17α-hydroxypregnanolone, and was capable of inhibiting to varying degrees the DOC-induced salt retention in adrenalectomized rats.

Small doses of progesterone can cause salt loss by inhibition of the salt-retaining effect of desoxycorticosterone and aldosterone in adrenalectomized animals (35), as well as in normal subjects (36) and addisonian patients treated with DCA (28, 29, 35, 37). Administration of large doses to rats, however, results in salt retention (35). Moreover, in humans, glucocorticoids can antagonize the sodium-retaining effects of aldosterone and DOC (5, 38, 39). This phenomenon was also observed with prednisone in the adrenalectomized, DCA-treated patient (I.S.) in the authors' series. These substances are probably not related to the salt loss seen in congenital adrenal hyperplasia, since progesterone and the glucocorticoids are not characteristically present in excessive amounts, as indicated by their urinary concentrations and those of their metabolites.

In the salt-losing form of congenital adrenal hyperplasia, ACTH increases or produces salt loss even when DCA is administered concurrently (14). This is associated with a rise in the urinary concentrations of pregnanetriol and 17-ketosteroids. It would appear, then, that the additional natriuresis stimulated by ACTH may be related to the enhanced elaboration by the adrenal cortex of 17α-hydroxyprogesterone, which, because of the 21-hydroxylase block, cannot be converted along normal pathways to compound S and hydrocortisone. Cortisone (and other glucocorticoids) ameliorates the salt loss and the excessive excretion of pregnanetriol and 17-ketosteroids (13, 40) by inhibiting the pituitary secretion of ACTH. This presumably reduces the circulating quantity of 17α-hydroxyprogesterone, which, if active initially in stimulating salt loss, reaches a level inadequate to promote saluresis.

Similarly, in patients with congenital adrenal hyperplasia who are not salt-losers, the absence of salt loss would appear to be related to a less pronounced 21-hydroxylase block. This would prevent sufficient accumulation of 17α-hydroxyprogesterone to overcome the efficacy of circulating salt-retaining steroids. The necessity for administration of relatively large amounts of 17α-hydroxyprogesterone to produce salt loss in "normal" subjects would imply that a balance exists between circulating 17α-hydroxyprogesterone and salt-retainers, whereby salt loss does not become manifest until a critical ratio between these two substances is exceeded.

Acknowledgment

The assistance of Miss M. E. Kalin and Miss C. Hill in the determination and maintenance of the balance diets is gratefully acknowledged.

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METHOD FOR THE SIMULTANEOUS DETERMINATION OF PREGNANEDIOL AND PREGNANETRIOL: ESTIMATION OF URINARY EXCRETION IN NORMAL SUBJECTS AND IN TEN PATIENTS WITH ADRENOCORTICAL DISORDERS*

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ABSTRACT

A new paper chromatographic method for the simultaneous determination of pregnanediol and pregnanetriol is described. Results compare favorably with values obtained by other procedures. In 15 normal subjects and 4 women with hirsutism, urinary excretion of pregnanediol and pregnanetriol paralleled the excretion of 17-OH-CS and 17-KS in the basal state and following stimulation with ACTH. In some patients with adrenal dysfunction, pregnanediol and pregnanetriol excretion varied independently of 17-OH-CS and 17-KS excretion, suggesting a derangement in steroid biosynthesis. Two patients with Cushing's syndrome caused by an adrenal adenoma showed normal pregnanediol and pregnanetriol excretion, but elevated 17-OH-CS with variable 17-KS excretion; this pattern might prove helpful in differentiating adenoma from other causes of Cushing's syndrome.

PREGNANEDIOL[1] was first isolated from the urine of pregnant women (1). It soon became apparent, however, that not all urinary pregnanediol could be accounted for by activity of the corpus luteum or placenta. Engel, Thorn and Lewis identified pregnanediol in the urine of nor-

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[1] The chemical names of the steroid compounds used in this study are: pregnane-3α,20α-diol (pregnanediol); pregnane-3α,17α,20α-triol (pregnanetriol); 4-pregnene-3,20-dione (progesterone); 17α-hydroxy-4-pregnene-3,20-dione (17-hydroxyprogesterone); 4-pregnene-21-ol,3,20-dione (desoxycorticosterone).

mal males, and suggested that it might be derived from the adrenal cortex (2). The absence of the normal rise in pregnanediol excretion following corticotropin (ACTH) stimulation in adrenalectomized patients has confirmed this hypothesis (3, 4).

Although pregnanetriol was first isolated from the urine of women with congenital adrenal hyperplasia (5), it has recently been identified in small quantities in the urine or normal men and women, and has been found to increase following stimulation with ACTH (4, 6, 7).

Pregnanediol and pregnanetriol are now considered metabolites of intermediaries along the pathway of adrenal steroid biosynthesis (8). Pregnanediol is believed to be derived primarily from adrenocortical progesterone (3, 9), and to a lesser extent from desoxycorticosterone (10). Pregnanetriol is considered to be a metabolite of 17-hydroxyprogesterone (8, 11).

Abnormal excretion of pregnanediol and pregnanetriol has been observed in patients with adrenocortical tumors (11–13), congenital adrenal hyperplasia (14–18), and in some women with hirsutism (19). In Cushing's syndrome, both normal and excessive levels have been reported in the resting state and following stimulation with ACTH (4, 7, 20).

The present investigation was undertaken to study the effect of exogenous ACTH upon the urinary excretion of pregnanediol and pregnanetriol in patients with a variety of adrenal disorders. Urinary 17-hydroxycorticosteroids (17-OH-CS) and 17-ketosteroids (17-KS) were also measured, and the results were correlated with pregnanediol and pregnanetriol excretion.

PROCEDURE

Subjects

Urine was collected in 24-hour specimens from 6 patients with Cushing's syndrome, and 4 patients with the adrenogenital syndrome. The pathologic diagnoses, established later at operation, were as follows:

Patient	Sex & Age		Clinical diagnosis	Pathologic diagnosis
S.D.	F	41	Cushing's syndrome	Adrenal carcinoma
P.P.	F	34	Cushing's syndrome	Adrenal hyperplasia
J.S.	M	19	Cushing's syndrome	Adrenal hyperplasia
P.R.	F	47	Cushing's syndrome	Adrenal hyperplasia
A.H.	F	42	Cushing's syndrome	Adrenal adenoma
F.F.	F	37	Cushing's syndrome	Adrenal adenoma
D.L.	F	18	Adrenogenital syndrome	Adrenal hyperplasia
E.B.	F	22	Adrenogenital syndrome	Adrenal hyperplasia
D.S.	F	34	Adrenogenital syndrome	Adrenal adenoma
L.M.	F	34	Adrenogenital syndrome	Adrenal carcinoma

Four women with idiopathic hirsutism (ages, 25 to 43) were also studied. Fifteen normal volunteers (6 males and 9 females) served as controls.

Urine collections were continued during a two-day ACTH test, with 25 units of ACTH infused intravenously over an eight-hour period on two successive days (21).

The completeness of the urine collections was checked by determinations of urinary creatinine. In a few instances, collections were incomplete and were discarded.

Analytical methods

Urinary 17-OH-CS were determined by the method described by Reddy (22). 17-KS were measured by a modification of the method of Drekter and associates (23).

A chromatographic method for the simultaneous determination of pregnanediol and pregnanetriol was developed for this study. All estimations were carried out in duplicate; the data recorded represent the mean value for the duplicate determinations.

Method for pregnanediol plus pregnanetriol. A 25-ml. aliquot of a 24-hour urine collection is adjusted to pH 5.0. Five ml. of 0.1 *M* acetate buffer, and 5 ml. of β-glucuronidase (Ketodase, Warner Chilcott, New York) are added, and the mixture is incubated at 37° C. for forty-eight hours. The steroids are extracted from the urine with 35 ml. of methylene dichloride. The methylene dichloride extract is washed with 5 ml. of 1.0 *N* sodium hydroxide followed by 5 ml. of distilled water, and then dried by the addition of 2 Gm. of anhydrous sodium sulfate. A 28-ml. sample of the methylene dichloride extract is acidified with a drop of glacial acetic acid, and evaporated to dryness under reduced pressure at 60° C. The residue and two standards each of pregnanediol and pregnanetriol are applied quantitatively to Schleicher and Shüll paper No. 2041, and chromatographed in the B-3 system of Bush (24). Localization of standards, elution, and measurement of sulfuric-acid chromogens are carried out by the methods of Bongiovanni and Eberlein (25, 26).

In sulfuric acid-bisulfite mixture, the absorption peak of pregnanediol occurs at 425 mμ; in concentrated sulfuric acid, the absorption peak of pregnanetriol occurs at 440 mμ. Paper blanks and reagent blanks show no absorption at these wave lengths when the Allen correction factor (27) is used.

With the use of the chromatographic method described, the recovery of 20 μg. of pregnanediol standard was 81 ± 11.5 per cent in 38 estimations; the recovery of 10 μg. of pregnanetriol standard was 76 ± 10.5 per cent in 48 determinations. The recovery of known amounts of pregnanediol and pregnanetriol which were added to urine and then extracted, was the same as the recovery when pure standards were added directly to the paper. The minimal amount of steroid detectable is approximately 250 μg. per twenty-four hours; however the sensitivity can be increased by using larger quantities of urine for extraction. The mean difference between duplicate determinations of 24-hour excretions was 0.42 mg. ± 0.48 S.D. for pregnanediol, and 0.3 mg. ± 0.40 S.D. for pregnanetriol.

RESULTS

Normal subjects (Table 1)

Values for the 24-hour urinary excretion of 17-OH-CS, 17-KS, pregnanediol, and pregnanetriol in 15 normal subjects are shown in Table 1. Mean levels and mean increments following ACTH stimulation have been calculated for males and females. The values for pregnanediol and pregnanetriol compare well with those obtained by other procedures (3, 4, 16, 28, 29).

Cushing's syndrome (Table 2)

All patients with Cushing's syndrome had a high baseline (control) excretion of 17-OH-CS and 17-KS, and all showed an excessive response to

TABLE 1. URINARY STEROID EXCRETION* IN NORMAL SUBJECTS (AGE RANGE, 20–40 YEARS)

Subjects	Pregnanediol (mg./24 hrs.)			Pregnanetriol (mg./24 hrs.)			17 Ketosteroids (mg./24 hrs.)			17-OH-corticoids (mg./24 hrs.)		
	Control	Following ACTH		Control	Following ACTH		Control	Following ACTH		Control	Following ACTH	
		Day 1	Day 2		Day 1	Day 2		Day 1	Day 2		Day 1	Day 2
Women												
B.R.	4.9	6.1	6.8	1.9	2.7	4.3	9.0	17.2	27.2	6.7	20.3	41.4
J.R.	0.6	1.2	2.2	0.4	0.5	1.4	5.8	13.1	20.4	4.3	24.9	35.3
A.H.	1.2	3.9	3.4	0.8	2.5	2.3	5.3	13.5	22.4	6.0	31.7	35.2
M.A.	0.2	0.9	2.7	0.2	1.3	3.5	5.2	18.0	23.2	6.3	17.6	31.9
J.L.	4.2	6.1	6.3	2.1	6.4	6.2	9.5	21.4	30.2	8.9	27.1	32.7
S.L.	0.9	1.8	2.1	0.7	1.6	2.2	4.5	11.1	15.9	5.6	23.7	36.3
N.M.	0.3	2.9	3.1	0.4	2.4	3.8	5.2	16.8	21.0	6.2	24.6	40.4
A.E.	3.8	6.3	7.0	2.0	3.8	5.2	12.9	30.0	39.2	2.0	24.7	38.8
J.V.	1.2	3.9	5.4	0.7	2.6	3.4	7.6	17.1	17.4	3.9	23.1	24.9
Mean increment**	1.9	3.7 1.8	4.3 2.4	1.0	2.6 1.6	3.6 2.6	7.2	17.6 10.4	24.1 16.9	5.6	24.2 18.6	35.2 29.6
Men												
W.H.	1.9	2.4	4.5	1.7	2.7	5.3	20.0	24.0	44.0	6.6	24.0	45.5
R.D.	2.1	4.1	3.6	1.4	2.8	3.2	20.9	26.4	29.4	2.0	20.7	26.1
A.L.	1.8	3.6	4.5	1.1	2.3	3.2	17.1	26.2	28.0	1.9	19.4	29.0
E.R.	1.3	2.0	6.5	0.9	3.5	6.8	11.0	30.2	32.3	6.1	25.7	33.5
J.B.	0.6	1.3	3.2	0.6	2.0	3.6	3.2	26.6	36.7	6.0	29.7	38.7
J.C.	0.4	2.5	4.1	0.6	3.6	4.4	17.6	26.0	32.7	6.1	26.6	31.3
Mean increment**	1.4	2.7 1.3	4.4 3.0	1.1	2.8 1.7	4.4 3.3	15.8	26.6 10.8	33.9 18.1	4.8	24.4 19.6	34.0 29.2

* Mean values of duplicate estimations.
** Increment above mean control values.

ACTH stimulation, with the exception of 1 of the 2 patients with adrenal adenomas. This patient had normal 17-KS excretion in both the control period, and following ACTH stimulation.

Pregnanediol and pregnanetriol excretion was elevated only in the patient with an adrenal carcinoma; patients with adrenal hyperplasia or adrenal adenomas showed normal values, in keeping with those reported by others (4, 7). In the patients with adrenal hyperplasia there was an increase in the excretion of pregnanediol and pregnanetriol following stimulation with ACTH, but in both patients with adrenal adenoma the excretion was normal following ACTH.

Adrenogenital syndrome (Table 2)

All 4 patients showed a normal or low excretion of 17-OH-CS and an elevated excretion of 17-KS in the basal state. Following administration of ACTH, both patients with adrenal hyperplasia, although differing in the 17-OH-CS response, showed the expected abnormal rise in 17-KS excretion. In the patient with adrenal carcinoma the 17-OH-CS response was poor but the 17-KS response was normal following administration of ACTH.

Control values for pregnanediol and pregnanetriol were obtained in only 1 of the 2 patients with adrenal hyperplasia; a fivefold elevation in both steroid metabolites was found. Both patients exhibited excessive pregnanediol and pregnanetriol excretion following stimulation with ACTH.

TABLE 2. URINARY STEROID EXCRETION* IN PATIENTS WITH ADRENOCORTICAL DISORDERS, AND IN HIRSUTE WOMEN

Patient	Sex & age	Diagnosis	17-Ketosteroids (mg./24 hrs.)			17-OH-corticoids (mg./24 hrs.)			Pregnanediol (mg./24 hrs.)			Pregnanetriol (mg./24 hrs.)		
			Control	Following ACTH		Control	Following ACTH		Control	Following ACTH		Control	Following ACTH	
				Day 1	Day 2		Day 1	Day 2		Day 1	Day 2		Day 1	Day 2
		Cushing's syndrome:												
S.D.	F 41	Adrenal carcinoma	128.0	—	—	153.0	—	—	15.9	—	—	11.0	—	—
P.P.	F 34	Adrenal hyperplasia	22.5	58.5	67.0	16.5	39.5	61.3	—	3.3	14.4	—	5.4	12.0
J.S.	M 19	Adrenal hyperplasia	24.5	63.1	49.8	21.7	81.6	108.6	2.0	9.5	12.6	2.2	9.0	15.6
P.R.	F 47	Adrenal hyperplasia	17.0	41.0	47.4	32.3	52.5	69.5	1.8	6.8	12.7	1.3	4.5	14.0
A.H.	F 42	Adrenal adenoma	12.6	19.4	18.5	11.4	37.9	55.3	1.4	2.4	2.5	0.2	0.4	1.6
F.F.	F 37	Adrenal adenoma	18.7	31.4	57.8	19.3	30.0	58.0	2.8	4.7	4.4	2.5	3.3	4.8
		Adrenogenital syndrome:												
D.L.	F 18	Adrenal hyperplasia	24.2	74.6	71.7	5.3	31.2	49.0	—	9.9	8.2	—	13.0	11.0
E.B.	F 22	Adrenal hyperplasia	38.3	60.7	80.5	1.1	5.4	8.1	9.1	18.4	24.4	21.7	14.5	17.6
D.S.	F 34	Adrenal adenoma	394.0	—	—	6.3	—	—	7.9	—	—	11.2	—	—
L.M.	F 34	Adrenal carcinoma	20.6	34.4	41.0	1.2	11.2	11.9	2.2	4.3	4.4	1.6	2.5	3.3
P.W.	F 43	Hirsutism	8.3	—	—	6.3	—	—	1.8	—	—	2.0	—	—
J.F.	F 25	Hirsutism	8.9	23.7	25.9	4.2	19.6	28.8	1.7	4.2	4.6	1.7	2.0	2.7
G.W.	F 32	Hirsutism	9.9	20.4	20.8	8.0	31.4	30.6	0.9	3.7	3.7	1.7	3.8	4.4
M.J.	F 28	Hirsutism	20.4	37.0	41.2	3.6	19.6	29.0	1.4	3.1	3.4	1.5	3.8	4.0

* Mean values of duplicate determinations.

Pregnanediol and pregnanetriol excretion was normal both in the basal state and following ACTH stimulation in the patient with adrenal carcinoma. The patient with adrenal adenoma showed a high basal excretion of pregnanediol and pregnanetriol the effects of ACTH could not be studied in this case.

Hirsutism (Table 2)

The basal excretion of 17-KS was high in only 1 of the 4 women with hirsutism. Otherwise, all 4 women showed normal values for all the urinary steroids measured, both in the basal state and following administration of ACTH.

DISCUSSION

Following administration of ACTH to the normal subjects, there was an increase in all urinary steroids measured—17-OH-CS, 17-KS, pregnanediol and pregnanetriol. It seems reasonable to assume that increased steroid excretion reflects increased adrenocortical hormone production, since such increments have not been observed in adrenalectomized patients (3, 4). In certain disease states (Cushing's syndrome due to adrenal hyperplasia) the normal steroid excretion pattern is retained, but the response to ACTH is exaggerated. In other conditions it appears that the excretions of the various steroid metabolites may vary independently of each other. For example, patients with congenital adrenal hyperplasia show an increase in the excretion of pregnanediol, pregnanetriol and 17-KS, but no increase in 17-OH-CS excretion; this suggests an abnormality in steroid biosynthesis (30).

In Cushing's syndrome, the excretory patterns may prove sufficiently characteristic to be helpful in determining the causative lesion. In the 2 patients with Cushing's syndrome caused by an adrenal adenoma, the baseline excretion of pregnanediol and pregnanetriol was normal and remained normal following ACTH stimulation, *independently* of elevation in the excretion of 17-OH-CS and 17-KS. In the patients with Cushing's syndrome due to adrenal hyperplasia, excretion of pregnanediol and pregnanetriol was abnormally high following administration of ACTH. In one patient with adrenal carcinoma, baseline pregnanediol and pregnanetriol excretion was significantly elevated. The excretion of pregnanediol and pregnanetriol merits further investigation in patients with Cushing's syndrome.

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EFFECTS OF ORAL ADMINISTRATION OF ESTROGENS ON THE 17-KETOSTEROID EXCRETION OF VIRILIZED WOMEN*†

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ABSTRACT

Synthetic estrogens were given orally to virilized women with hirsutism and menstrual disorders, in amounts sufficient to suppress gonadotropin excretion to unmeasurable levels. In most subjects a significant and sustained reduction in urinary 17-ketosteroid excretion was observed both in patients with sclerocystic ovaries and in those with acquired adrenal virilism. In 2 patients receiving prolonged cyclic therapy, previously enlarged ovaries became impalpable, and in one case the reduced 17-ketosteroid excretion was maintained after therapy. Estrogen therapy significantly decreased urinary 17-ketosteroid excretion in an oophorectomized woman, but this effect was minimal in virilized subjects treated with prednisone. Accordingly, a functioning adrenal cortex seems necessary for the estrogen effect. A functioning ovary is not essential, but inhibition of its androgen secretion may result from estrogen therapy. Alteration in the secretion, plasma binding or metabolism of adrenal steroids is a more probable explanation for the reported observations.

HIRSUTISM, menstrual abnormalities and infertility comprise a common clinical triad, the endocrine basis of which is often obscure. Some women with this syndrome of mild virilism have sclerocystic ovaries (Stein-Leventhal syndrome) and the improvement in menstruation and fertility which may follow wedge resection indicates an ovarian source for the virilizing hormones. However, adrenal hyperfunction has been well documented in other patients with similar cystic ovaries (1–3); in these cases, even when total neutral 17-ketosteroid excretion is normal, the more potent androgens may predominate in fractional steroid analyses of urine (4–6). Accordingly, adrenal hypersecretion may be the primary defect in all cases. Suppression of the hypersecretion with glucocorticoids has been logically recommended (7).

The mechanism by which increased secretion of adrenal androgens might lead to cysts and disturbed function of the ovary has not been satisfactorily

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established. It is claimed that, in patients with congenital adrenal virilism, cortical hypersecretion can suppress pituitary gonadotropins with resulting secondary ovarian hypofunction (8). However, urinary gonadotropin levels are usually normal in patients with sclerocystic ovaries; in fact, McArthur and associates (9) have reported a consistent excess of the luteinizing (LH) fraction. Adrenal androgens, if etiologically important in this syndrome, probably do not affect ovarian function through pituitary suppression. In previous studies of virilized women we failed to demonstrate any correlation between urinary levels of 17-ketosteroids and pituitary gonadotropins (10).

This report is concerned with the clinical and laboratory results of endocrine treatment in patients with menstrual disorders, infertility, and mild virilism expressed primarily as hirsutism. Comparisons have been made between results obtained during 1) suppression of adrenocorticotropic (ACTH) and adrenocortical secretion by administration of glucocorticoids; 2) suppression of pituitary gonadotropin release and ovarian function by oral administration of estrogen; and 3) simultaneous suppression of both ACTH and gonadotropin.

METHODS

A modified Drekter method was used for determining urinary total 17-ketosteroids (11); their separation in selected specimens was accomplished by chromatography on alumina columns. The chromatographic method was that of Kellie and Wade (12), with separation of fractions containing dehydroisoandrosterone, androsterone, etiocholanolone and 11-oxygenated 17-ketosteroids. Urinary 17-hydroxycorticosteroids were measured in butanol extracts as Porter-Silber chromogens (13). Gonadotropic material, isolated from urine according to the method of Bradbury and Brown (14), was assayed in immature female white mice. Creatinine determinations were performed on all urine specimens to assess accuracy of the collections.

Patients were drawn from a group under study for various degrees of virilism usually associated with menstrual abnormalities and, in the married subjects, with infertility. In none of those selected was there evidence of advanced virilism such as clitoral hypertrophy, deepening of the voice, and pattern baldness.

In the cases classified as the Stein-Leventhal syndrome, subsequent operation revealed enlarged cystic ovaries with the characteristic thickened white ovarian capsule. These patients did not otherwise differ clinically from the group as a whole, with the exception that some had minimal hirsutism.

For suppression of adrenocortical secretion, prednisone (7.5 mg.) or methylprednisolone (6 mg.) was taken daily, two-thirds of the dose on retiring and the remainder early each morning. Duration of treatment ranged from two weeks to over one year. For suppression of ovarian secretion, ethinyl estradiol (1.0 to 1.5 mg. daily) or diethylstilbestrol (10–15 mg. daily) was administered orally for periods of three weeks to over one year. Dosages remained the same during periods of combined treatment with glucocorticoids and estrogen.

To assist in interpretation of results, data are cited from studies of 4 patients with

severe virilism, 3 of whom had congenital adrenal hyperplasia, and 1, a large benign adrenocortical adenoma.

RESULTS

Effect of glucocorticoid therapy

In Figure 1 are shown data for urinary 17-ketosteroid and pituitary gonadotropin excretion in 15 patients with the Stein-Leventhal syndrome. All values are averages of multiple but not necessarily serial determinations. Urinary 17-ketosteroid excretion was normal (8–15 mg. per twenty-four hours) in 8 subjects, and was from 2 to 10 mg. above normal in the remaining 7 subjects. Pituitary gonadotropin titers, likewise variable, were normal (8–32 mouse units per twenty-four hours) and no correlation was found between the values for the two indices.

In all subjects given glucocorticoids orally the expected reduction in 17-ketosteroid excretion was observed. Although some concomitant change was observed in gonadotropin titers in the subjects with suppressed adrenals, the direction of the change was inconsistent. In fact, the average titer for each patient during glucocorticoid therapy was the same—16 mouse uterine units.

Effect of estrogen therapy

Nine women with hirsutism and abnormal menses were given estrogen alone by the oral route (Fig. 2). Enlarged sclerocystic ovaries were found at

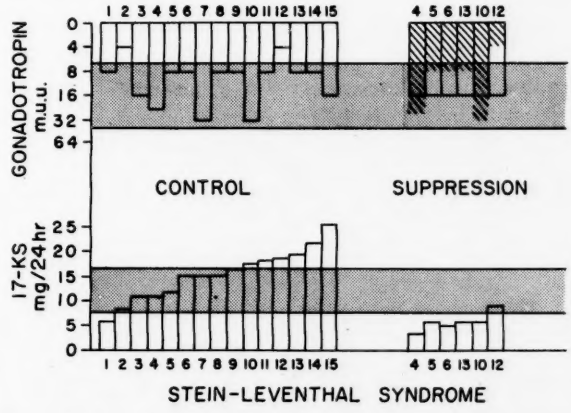

FIG. 1. Urinary 17-ketosteroid and gonadotropin levels in 15 women with sclerocystic ovaries. Shaded area represents normal range. There was no correlation between the two indices. During the period of suppressed adrenocortical function (by glucocorticoid therapy), the gonadotropin level was 16 units in all 6 subjects, representing a reduction in 2 and an increase in 4 subjects.

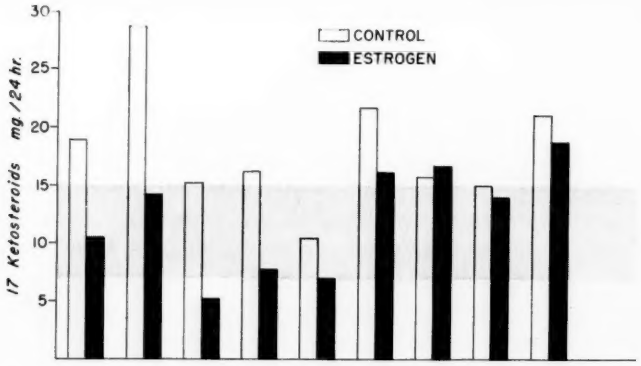

FIG. 2. Effect of estrogen on 17-ketosteroid excretion in 9 mildly virilized women. Shaded area represents the normal range. All subjects had sclerocystic ovaries except the seventh (reading from the left), who had adrenal hyperplasia.

laparotomy in all but one patient, the seventh case. The primary disorder in the second case was acquired adrenal virilism, as manifested by a basal 17-ketosteroid secretion of 28–29 mg. per twenty-four hours and an exaggerated response to ACTH stimulation. The seventh woman, the single subject without sclerocystic ovaries, had bilateral adrenal hyperplasia (demonstrated at operation). Adrenal function was considered normal in the others. During the period of treatment with estrogen, gonadotropin titers fell to less than 4 mouse uterine units in all 24-hour urine specimens. In 6 subjects, urinary 17-ketosteroid excretion was reduced 25 to 60 per cent below the control values, but in 3 patients no significant reduction occurred. The mean and standard error of the control 17-ketosteroid excre-

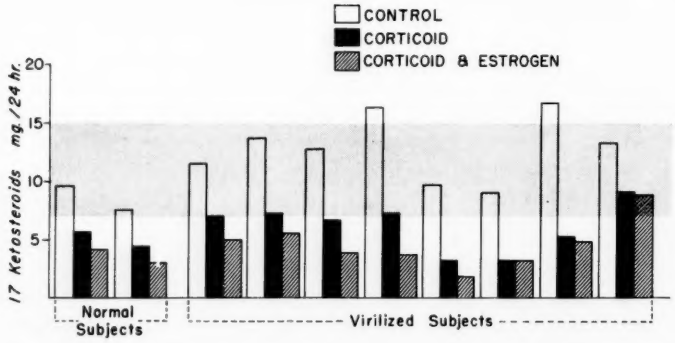

FIG. 3. Effect of estrogen on urinary 17-ketosteroid levels of normal and virilized women receiving adrenal-suppressing glucocorticoid therapy. Levels were further reduced in 7 of 10 subjects.

tion values for the entire group was 18.2 ± 1.7 mg. per twenty-four hours. This value fell to 12.7 ± 1.7 mg. during therapy with estrogen ($P = .05 - .02$).

Effects of combined estrogen and corticosteroid therapy

The results shown in Figure 3 were obtained in 8 mildly virilized and 2 normal young women when estrogen therapy was superimposed on corticosteroid therapy. Prednisone produced a significant reduction of 17-ketosteroid levels in all 10 subjects. The addition of estrogen produced further suppression in 7 (including the 2 normal women), but no change in the remaining 3 subjects. The data represent results of 69 separate determinations. The mean and standard error of the 17-ketosteroid excretion values for the entire group during the control period was 12.0 ± 0.9 mg. per twenty-four hours. With corticoid therapy alone it was 6.0 ± 0.6 mg., and with corticoid plus estrogen it was 4.4 ± 0.6 mg. Although the effect of added estrogen is of questionable significance when this group of patients with suppressed adrenal function is considered as a unit, the evidence for further reduction of 17-ketosteroid excretion in some patients is undeniable.

In Figure 4 are presented the results of administration, first of ethinyl estradiol and then of prednisolone, to an extremely hirsute 20-year-old woman with surgically demonstrated polycystic ovaries. The patient re-

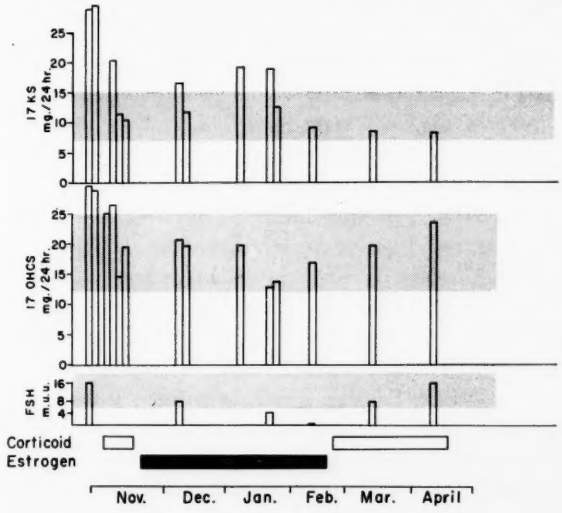

FIG. 4. Urinary steroid and gonadotropin excretion in a markedly hirsute woman with adrenal hyperfunction and sclerocystic ovaries. 17-Ketosteroid excretion values during estrogen therapy were less than half of the control values and gonadotropins became undetectable.

ported an interval of amenorrhea in her midteens; despite the ovarian pathology, however, regular ovulatory menses occurred for two years before, and during the period of study. The distinctly increased urinary 17-ketosteroids (28–29 mg. per twenty-four hours) and the sharp adrenocortical response to corticotropin stimulation (not shown in the figure) were consistent with a primary diagnosis of acquired adrenal hyperfunction. The excretion of 17-ketosteroids fell promptly during a brief period of corticosteroid administration and then was maintained at approximately half the control value (14 mg.) during prolonged estrogen therapy. By comparison, subsequent corticoid therapy suppressed the level to 8 mg. daily. Urinary gonadotropins disappeared during estrogen therapy and returned to control values when corticoids alone were administered. Administration of glucocorticoids was continued for approximately one year, during which time the hirsutism diminished and the patient continued to have regular ovulatory menses. Two months after all therapy was discontinued, urinary 17-ketosteroid excretion remained at a level of 13 mg. per twenty-four hours.

In Figure 5 are illustrated graphically the absolute values as well as the relative proportions of urinary dehydroepiandrosterone (DHA), androsterone, etiocholanolone and 11-oxygenated 17-ketosteroids excreted by the foregoing patient during both control and treatment (estrogen or glucocorticoid) periods. All four fractions of 17-ketosteroids were increased, DHA particularly, and all fractions were diminished during the separate treatments. DHA was decreased to a proportionately greater degree than the other components, particularly during prednisone therapy.

The data shown in Figure 6 were recorded in the study of a 27-year-old woman with hirsutism, menstrual irregularity and infertility. The elevated 17-ketosteroid excretion of 20 mg. per twenty-four hours was sharply reduced during four days of prednisone therapy, and was maintained at approximately 10 mg. per twenty-four hours during six weeks of estrogen therapy alone. Following the withdrawal of estrogen, 17-ketosteroid excretion returned to control levels, but subsequent oral administration of prednisolone reduced the level to 6 mg. per twenty-four hours. With the addition of ethinyl estradiol, the 24-hour excretion of 17-ketosteroids reached the low level of 1.5 mg. Gonadotropins, unaltered by corticoid therapy, were suppressed by estrogen. After nearly a year of glucocorticoid therapy, all hormone administration was discontinued and 17-ketosteroid excretion remained normal (8–10 mg. per twenty-four hours). Although hirsutism was benefited, the irregular and anovulatory menses were not improved. Therefore, laparotomy was performed. Enlarged ovaries with typical sclerocystic lesions were found.

Illustrated in Figure 7 are the urinary 17-ketosteroid fractions for this patient before, during and after therapy with prednisone, and while

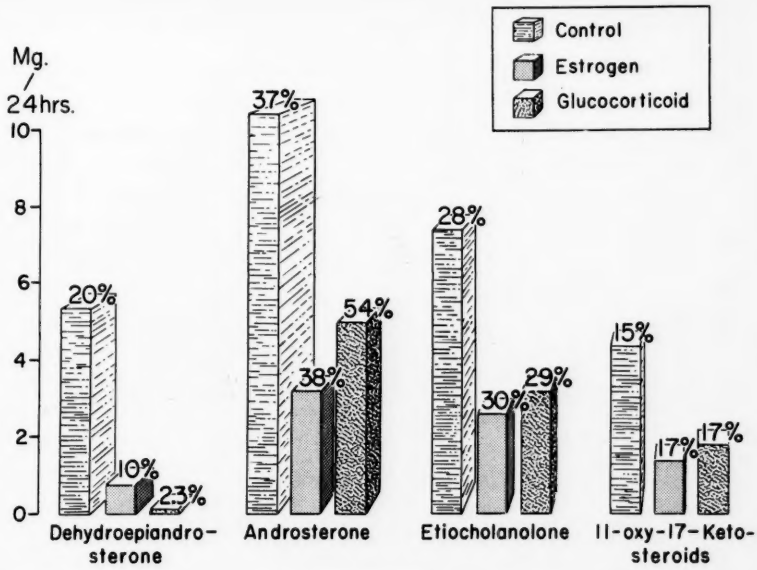

FIG. 5. Urinary 17-ketosteroid fractions for the subject represented in Figure 4. Height of column indicates absolute amount of each fraction, and the overlying figure is the percentage of total 17-ketosteroid for each fraction. The markedly increased DHA fraction underwent the greatest reduction during both treatment periods.

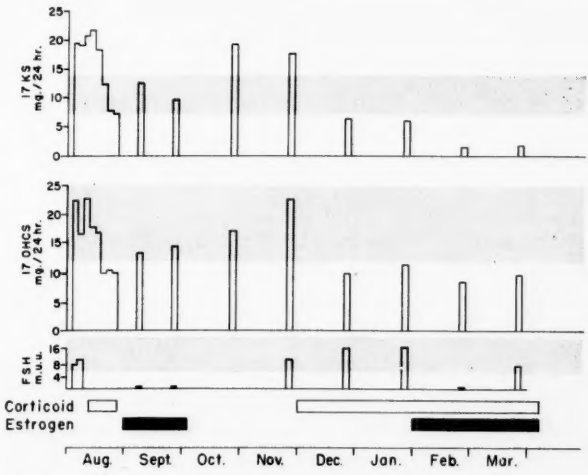

FIG. 6. Effects of administered corticoid and estrogen in a subject with the Stein-Leventhal syndrome. Each hormone significantly suppressed urinary steroid excretion and, when combined, further reduced 17-ketosteroids to very low levels (1.5 mg. per twenty-four hours).

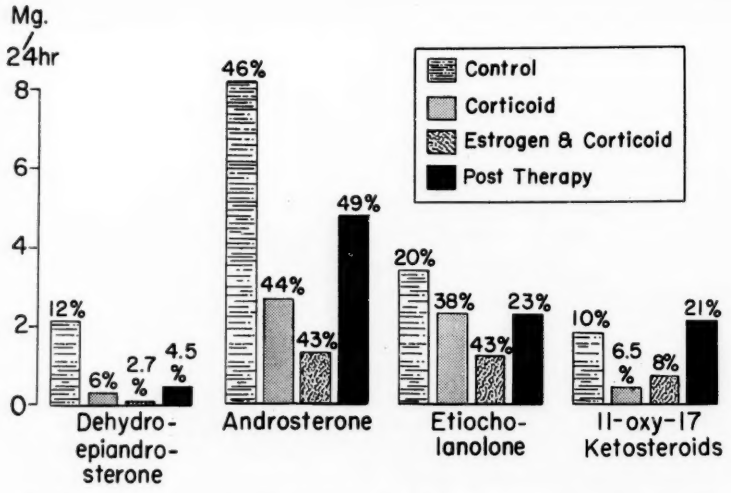

FIG. 7. Urinary 17-ketosteroid fractions for the subject represented in Figure 6. There was a reduction in all fractions during therapy. Post-therapy values remained significantly less than control values. Androsterone, comprising nearly half of each 17-ketosteroid fraction, was not preferentially suppressed.

estrogen was concurrently administered. The androsterone fraction predominated in all specimens. Corticoid therapy suppressed all fractions and the addition of estrogen enhanced this suppression. DHA excretion was reduced proportionately more than were the other fractions during both treatment periods. After therapy was discontinued all fractions remained below control levels, with androsterone still constituting nearly half the total excretion.

Observations on patients with severe virilism

In Figure 8 are shown values for the urinary 17-ketosteroid and gonadotropin excretion in 3 women with congenital virilizing adrenal hyperplasia. In none of the 3 had feminine secondary sex characteristics developed, and none had ever menstruated. However, gonadotropins had not been suppressed by the cortical hyperfunction, and cortisone therapy failed to increase the titers, even though improved ovarian function, feminization and menstruation resulted from the treatment. Urinary gonadotropin titers in a fourth patient, severely masculinized from an adrenal adenoma, were consistently greater than 64 mouse uterine units, and 17-ketosteroid excretion approached 2 grams daily. Removal of the tumor resulted in regular ovulatory cycles, a normal pregnancy and ultimately the birth of a normal female infant.

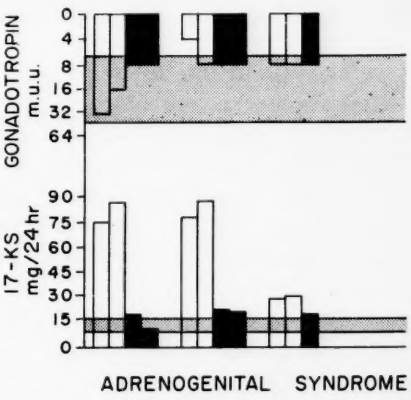

FIG. 8. Urinary excretion of 17-ketosteroids and gonadotropin by 3 adult women with congenital adrenal hyperplasia. White bars indicate the values before treatment, and black bars the values during glucocorticoid therapy. Gonadotropins were not suppressed by the high endogenous secretion of steroid, and adrenal suppression did not increase gonadotropin titers although feminization occurred.

DISCUSSION

Young women with hypertrichosis, menstrual disorders and infertility, despite resembling each other clinically, undoubtedly do not represent an endocrine entity. Although many such patients have sclerocystic ovaries and are clinically improved by wedge resection of the enlarged gonads, even this group presents marked variations in glandular functions. It is still to be confirmed that either abnormal secretion of pituitary gonadotropins or of adrenocortical steroids is the pathogenetic basis of the Stein-Leventhal syndrome. The reported successful management of the clinical problem by adrenal-suppressing glucocorticoid therapy suggests that abnormal or excessive adrenocortical secretion accounts for the disorder (15). However, our experience has been that most patients with the Stein-Leventhal syndrome have normal adrenocortical function, as demonstrated by 1) normal levels of urinary 17-hydroxysteroids and 17-ketosteroids, 2) normal increments of these steroids in response to ACTH, 3) normal decrements in response to administered glucocorticoids, and 4) normal levels of urinary pregnanetriol (16). When glucocorticoid therapy restores regular ovulatory cycles in these patients, abnormal adrenocortical function cannot necessarily be implicated as a cause of the virilizing disorder. A pronounced increase in urinary gonadotropin levels has been observed in patients, endocrinologically normal, treated with large doses of corticosteroids which suppress adrenal function. However, similar effects are also produced by corticotropin therapy, which markedly enhances adrenal secretion (17). Glucocorticoids given to virilized women may possibly affect gonadotropic secretion directly, thereby altering ovarian function. Although urinary gonadotropin titers were quantitatively normal in our mildly virilized patients, qualitative variations in gonadotropin activity have not been excluded, since only one assay method was used. Nevertheless, it is clear that urinary gonadotropins are not suppressed in these

patients, even when 17-ketosteroid excretion is elevated above normal. The claim that gonadotropins are suppressed by masculinizing adrenal steroids must be questioned, in view of our observations (Fig. 8) in patients with marked adrenal virilism. A disturbance of the pituitary-ovarian relationship secondary to adrenocortical dysfunction is an unlikely explanation for the Stein-Leventhal syndrome.

Estrogen, in doses large enough to suppress gonadotropin excretion in these virilized patients, induced a significant reduction of urinary 17-ketosteroid excretion. This suggests an ovarian source for the androgenic hormones. Direct evidence of androgen synthesis by sclerocystic ovaries incubated *in vitro* with chorionic gonadotropin has been reported recently by Lanthier (18). This attractive hypothesis is supported by our clinical observations on estrogen-treated patients in whom prolonged therapy reduced hypertrichosis. In 2 such cases, previously enlarged ovaries were not palpable during therapy; and in the one patient available for post-treatment study, the reduced 17-ketosteroid excretion did not return to the initial high levels.

Even if this hypothesis be correct, estrogen inhibition of pituitary-ovarian function is probably not the only means whereby 17-ketosteroid excretion is reduced. Direct evidence that other mechanisms are involved was provided by studies of the effects of ethinyl estradiol in a 30-year-old ovariectomized woman. Urinary 17-ketosteroid excretion was reduced from 15.5 to 6.0 mg. per twenty-four hours, suggesting an alteration either of the secretion or metabolism of adrenal steroids. Other investigators have reported that plasma levels of free 17-hydroxycorticosteroids are significantly higher during estrogen administration and normal pregnancy (19). This increase and the associated reduction in the urinary excretion of corticoids are attributed to enhanced cortisol-binding by plasma proteins, holding free steroids in a metabolically inactive state (20). The rates both of corticoid synthesis by the adrenal and of metabolic removal of infused cortisol are reduced during estrogen therapy (21). Similar alterations in plasma binding and metabolism of 17-ketosteroids have been proposed. Herrmann *et al.* (22) have shown a significant reduction in all 17-ketosteroid fractions in the urine of normal men and women receiving estrogen, even with doses which are generally smaller than those reported in other studies. Increased plasma levels of free cortisol were not observed. The authors concluded that the reduced urinary 17-ketosteroid excretion probably was secondary to increased plasma-protein binding of the hormone precursors, since the urinary 17-ketosteroid response to administration of ACTH was also less in these estrogen-treated patients. Other observers, studying estrogen effects on adrenal steroid metabolism, have reported no significant changes in 17-ketosteroid excretion (19, 21).

The chromatographic analysis of the 17-ketosteroids in the urine of the

2 virilized patients reported here tend to confirm the results of Herrmann *et al.* (22). The patterns of the 17-ketosteroid fractions in the urine of the 2 patients differed from those in normal subjects and from each other. All fractions were reduced during administration of estrogen, either alone or in combination with prednisone. The reduction in urinary DHA seemed proportionately greater than that of the other steroids when estrogen was administered, but the difference was of questionable significance. The effects of prednisone were similar to those observed with estrogen, although suppression of DHA was more profound. Adrenocortical suppression can explain the changes produced by prednisone, but the mechanism by which estrogen produces its effects is not established. There are several explanations for the reduced urinary 17-ketosteroid excretion observed in most of the virilized subjects. Inhibition of ovarian secretion through gonadotropin suppression has already been suggested, as has the increase in plasma binding of adrenal androgens, with reduced rates of their metabolism and excretion. Interference with the hepatic metabolism of steroids may occur, as has been shown for cortisol in estrogen-treated subjects (20). There may be a reduction in the rate of adrenocortical secretion, either as a direct estrogen effect on adrenal steroid biogenesis, as reported by McKerns (23), or as a result of inhibited pituitary ACTH release caused by the prolonged increase of plasma cortisol concentration. Failure of estrogen to suppress urinary 17-ketosteroids in some patients is unexplained, but it appears unrelated to either basal excretion rates or the apparent source of the virilizing steroids. Limitations in the capacity of plasma for steroid binding may be postulated.

Acknowledgment

The authors wish to express their appreciation for the technical assistance of Miss Jalileh Mansour and Mrs. Sarah Marsh in this study.

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THE INDEPENDENT ESTIMATION OF 11-DESOXYCORTISOL AND CORTISOL IN A SINGLE PLASMA SAMPLE*

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ABSTRACT

A method for the determination of cortisol and 11-desoxycortisol in a single small sample of blood plasma is described. This column chromatographic method employs, successively, Florisil and silica gel columns for purification and separation of cortisol and 11-desoxycortisol. After separation of the steroids, each is measured quantitatively by use of the Porter-Silber reaction. Values were determined for cortisol and 11-desoxycortisol in the early morning blood plasma of normal subjects. The application of the method in conjunction with the SU-4885 test is illustrated by presentation of the results obtained in 2 normal subjects and in 2 patients with Cushing's syndrome due to adrenal hyperplasia.

ALTHOUGH methods for determining the concentration of 11-desoxycortisol in plasma are available, they all are dependent upon the use of paper chromatography. They require specialized equipment, are time-consuming, and in most instances require a large amount of plasma. To a considerable extent these disadvantages could be minimized by the use of column chromatography. Furthermore it has been shown that the use of column, rather than paper, chromatography in the determination of 17-hydroxycorticosteroids (17-OH-CS) does not entail a great sacrifice in accuracy (1). It seemed worth while to attempt to develop a satisfactory column-chromatographic method for estimating 11-desoxycortisol in plasma. The purpose of this communication is to report a method for separating and estimating the concentrations, independently, of 11-desoxycortisol and cortisol in a single small blood sample.

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MATERIALS AND METHODS

The steroids were separated by employing successively, with slight modification, column chromatography with Florisil as proposed by Nelson and Samuels (2) and with silica gel as proposed by Sweat (3). After resolution of the steroids, the concentrations of 11-desoxycortisol and of cortisol were estimated by the Porter-Silber reaction (4)

Apparatus

Macro-columns, patterned after the chromatographic column described by Nelson and Samuels (2).

Micro-columns, patterned after the chromatographic column described by Sweat (3).

Instruments. A Beckman DU spectrophotometer. Readings are taken at 370 mμ 410 mμ and 450 mμ.

Reagents

1. *Chloroform U.S.P.* Redistilled over 5 Gm. of anhydrous potassium carbonate, the first part of the distillate being discarded. Should be used within four hours after distillation.

2. *Methanol, A.R., absolute.* Redistilled over 2.5 Gm. of 2,4-dinitrophenylhydrazine, the first part of the distillate being discarded. Stored in brown glass containers.

3. *Diethyl ether, A.R. grade.* Distilled and stored in the cold.

4. *Acetone, A.R. grade.* Distilled and stored in the cold.

5. *62% Sulfuric acid.* C.P., reagent grade (Du Pont).

6. *Phenylhydrazine-HCl,* (Eastman Kodak). Recrystallized from absolute ethanol four times.

7. *Phenylhydrazine reagent*—16 mg. of reagent No. 6 dissolved in 10 ml. of reagent No. 5, prepared fresh on the day it is used (2).

8. *Florisil,* size 60–100 mesh, (the Floridin Company). Prepared and activated as described by Eik-Nes *et al.* (5).

9. *Silica gel,* through 200 mesh (Davison Chemical Corporation). Prepared and activated as described by Sweat (3).

10. *Powdered glass,* sizes 20–30, 30–40, 40–50 mesh (Corning).

Procedure

The plasma sample. Blood is withdrawn by venipuncture into a heparinized syringe. The plasma is separated as soon as possible and stored in the frozen state until the extraction procedure is started. For this purpse, a 5–10 ml. sample of plasma usually is adequate.

Extraction. The plasma sample is subjected to extraction three times with 20 ml. of chloroform. Extraction is accomplished with the aid of a glass stirring rod for mixing, and centrifugation for resolving chloroform and plasma layers. The chloroform layer is transferred with a serum lifter to an Erlenmeyer flask through a filter paper. The three extracts are combined and evaporated to dryness under a continuous air stream in a water bath (40°–50° C.).

Florisil-column chromatography. This column is prepared and run as described by Nelson and Samuels (2). The elution procedure differs in that, following development of the column with 25 ml. of chloroform, 4 per cent methanol in chloroform (25 ml.) and 15 per cent methanol in chloroform (45 ml.) are used. The latter is collected in Erlenmeyer flasks and evaporated to dryness as described under "Extraction."

Silica-gel column chromatography. This column is prepared and run as outlined by

Ely *et al.* (6), with slight modifications. The column is packed with dry silica gel rather than with a slurry. Chloroform (5 ml.) is used to wet down the silica gel. Fractions 3, 4 and 5 as described by Ely *et al.* (6) are combined and collected as "the 1 per cent methanol fraction," and their fractions 6 and 7 are combined and collected as "the 5 per cent methanol fraction." These two fractions are collected separately in flubes[1] and evaporated to dryness as described under "Extraction." The sides of these flubes are washed with chloroform three times and the contents evaporated to dryness to insure that all the residue is in the small wells.

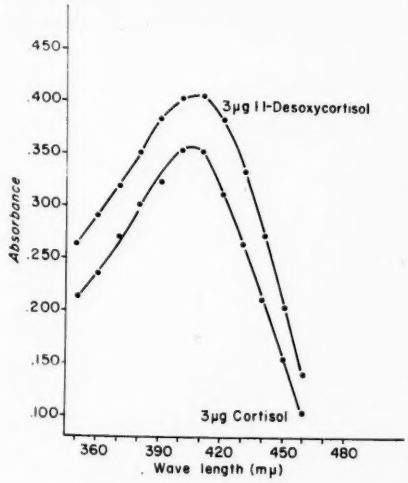

FIG. 1. Absorption spectra of cortisol and 11-desoxycortisol.

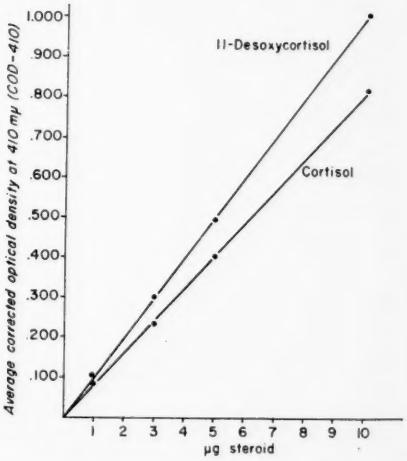

FIG. 2. Relationship of concentration of cortisol and 11-desoxycortisol to optical density (Beer's law).

Porter-Silber reaction. This is performed as described by Nelson and Samuels (2). Solvent blanks and standards always are run simultaneously with the samples. The samples are read at 370, 410 and 450 mµ, and the Allen (7) correction factor is applied to find the corrected optical density at 410 mµ (COD 410).[2] This is compared to the COD 410 of the standards for quantitation. The standards are 3 µg. of 11-desoxycortisol and 5 µg. of cortisol.

RESULTS

Porter-Silber reaction

Figure 1 shows the absorption curves for 3 µg. of cortisol[3] and 3 µg. of 11-desoxycortisol.[3] Figure 2 shows for each steroid the linear relationship of

[1] Flubes are 50-ml. round-bottom flasks, to the bottom of which 3-ml. glass wells are fused.

[2] COD 410 = 2×OD 410 − (OD 370 + OD 450).

[3] Standard steroids used in these studies were generously supplied by Dr. H. C. Peltier, The Upjohn Company, Kalamazoo, Mich.

absorbance to concentration expected in conformity with Beer's law. As is evident from Figures 1 and 2, the absorbance of 11-desoxycortisol is greater than that of cortisol. Gold *et al.* (8) noted a similar difference although not of the same magnitude.

Florisil-column chromatography

When varying concentrations of methanol in chloroform were used to determine which would give optimal recoveries of 11-desoxycortisol and cortisol from the columns, it was found that 81–88 per cent of the steroids

TABLE 1. RECOVERY OF CORTISOL AND 11-DESOXYCORTISOL FROM FLORISIL COLUMNS, USING VARYING CONCENTRATIONS OF METHANOL IN CHLOROFORM

Per cent Methanol—Chloroform	Cortisol recovery (%)	11-Desoxycortisol recovery (%)
4	<1	<1
6	38	45
8	80	60
10	81	76
12	84	82
15	86	87
20	88	83
25	87	81

were recovered with methanol concentrations higher than 12 per cent (Table 1). With 15 per cent methanol in chloroform, nearly optimal recovery of both cortisol and 11-desoxycortisol was attained. Virtually no 11-desoxycortisol or cortisol was eluted in the 4 per cent methanol-chloroform fraction. This latter concentration was used to develop the column, as it was found to elute all 17-hydroxyprogesterone, which previously has been observed (9) to interfere with the determination of 17-hydroxycorticosteroids by the Porter-Silber reaction.

Silica-gel column chromatography

Ely *et al.* (6) showed that cortisol is eluted in the 5 per cent methanol-chloroform fraction. The present study confirms this work and also shows that 11-desoxycortisol is eluted in the 1 per cent methanol-chloroform fraction (Table 2). As shown in Table 2, mixtures of 11-desoxycortisol and cortisol were separated satisfactorily. Even when the individual steroids were present in various proportions, with the concentration of each varying from 1 to 10 μg., good separation was achieved and the recovery of each steroid was satisfactory.

Combined column chromatography

When Florisil and silica-gel chromatography were used successively, the results were very similar to the foregoing findings, although the final re-

covery was lower. With increasing experience, the recoveries improved and at present are consistently 70 ± 5 per cent. Any variation in recovery is compensated for in calculating the steroid values for the unknown sample by comparing the COD 410 of the unknown sample with the COD 410 of the standards which are run with each series of determinations.

Recovery of steroid added to plasma

In order to evaluate the method, various amounts and mixtures of pure 11-desoxycortisol and cortisol were added to outdated stored blood-bank

TABLE 2. RECOVERY AND SEPARATION OF CORTISOL AND 11-DESOXYCORTISOL FROM SILICA-GEL COLUMNS

Sample	1% Methanol-Chloroform		5% Methanol-Chloroform	
	COD 410	Recovery (%)	COD 410	Recovery (%)
1 μg. 11-desoxycortisol	0.061	60	0	0
3 μg. 11-desoxycortisol	0.226	75	0	0
5 μg. 11-desoxycortisol	0.368	74	0	0
10 μg. 11-desoxycortisol	0.825	77	0	0
1 μg. cortisol	0	0	0.060	72
3 μg. cortisol	0	0	0.154	65
5 μg. cortisol	0	0	0.137	95
10 μg. cortisol	0	0	0.654	80
1 μg. 11-desoxycortisol + 1 μg. cortisol	0.064	66	0.056	72
1 μg. 11-desoxycortisol + 3 μg. cortisol	0.066	70	0.185	79
1 μg. 11-desoxycortisol + 5 μg. cortisol	0.062	68	0.273	71
1 μg. 11-desoxycortisol + 10 μg. cortisol	0.075	80	0.672	86
3 μg. 11-desoxycortisol + 1 μg. cortisol	0.251	81	0.078	95
5 μg. 11-desoxycortisol + 1 μg. cortisol	0.420	81	0.091	110
10 μg. 11-desoxycortisol + 1 μg. cortisol	0.914	87	0.073	90
10 μg. 11-desoxycortisol + 10 μg. cortisol	0.891	86	0.780	95

plasma and the resultant steroid concentrations were determined. Table 3 shows the results of these studies. The added steroids were completely separated and extracted from the plasma. The variation noted in the amounts of cortisol extracted from plasma, either before or after the addition of steroid, is attributable to the fact that outdated stored plasma was used. Gold *et al.* (8) observed this previously. When the concentration of total 17-hydroxycorticosteroids in the plasma was estimated by the Nelson-Samuels method, similar variations were noted.

Comparison to Nelson-Samuels method

The present method was compared with the Nelson-Samuels method —the standard procedure of measuring 17-hydroxycorticosteroids in our

TABLE 3. RECOVERY AND SEPARATION OF CORTISOL AND 11-DESOXYCORTISOL ADDED TO 10 ML. OF OUTDATED STORED BLOOD-BANK PLASMA, UTILIZING THE COMPLETE METHOD

Sample	1% Methanol-Chloroform		5% Methanol-Chloroform	
	COD 410	Average μg. 11-desoxycortisol	COD 410	Average μg. cortisol
Plasma	0.009		0.024	
	0.011	0.17	0.021	0.5
	0.010		0.024	
	0.011		0.021	
Plasma +3 μg. 11-desoxycortisol	0.186		0.053	
	0.184	3.10	0.043	0.80
	0.183		0.034	
	0.201		0.036	
Plasma +5 μg. cortisol	0.00		0.273	
	0.00	0.00	0.280	5.9
	0.00		0.305	
	0.00		0.312	
Plasma +3 μg. 11-desoxycortisol +5 μg. cortisol	0.197		0.276	
	0.171	3.0	0.307	6.0
	0.166		0.315	

laboratory—by using each to measure known amounts of 11-desoxycortisol and cortisol added to outdated stored blood-bank plasma. Table 4 shows the results of this experiment. The Nelson-Samuels method measures total free 17-hydroxycorticosteroids as cortisol equivalents, *i.e.*, cortisol is used as the standard from which the calculation of total 17-hydroxycorticosteroid concentration is made. The higher COD 410 per μg. of 11-desoxycortisol as compared with the COD 410 per μg. of cortisol will thus yield a higher value when the 11-desoxycortisol present in the sample is calculated as total 17-hydroxycorticosteroids in cortisol equivalents. In Table 4 the "experimental" values for total 17-hydroxycorticosteroids in the Nelson-Samuels method and for cortisol and 11-desoxycortisol in the present method are those obtained by the method involved. The "experimental" values for total 17-hydroxycorticosteroids in the present method are the sum of the values for cortisol and 11-desoxycortisol as obtained in the method but expressed as cortisol equivalents in order to be comparable to the Nelson-Samuels method. The "expected" values in all cases are those calculated from the known amounts of added steroid. Again, 11-desoxycortisol is expressed as cortisol equivalents in the calculation of "expected" 17-hydroxycorticosteroids, for comparison with the Nelson-Samuels method. The plasma values are considered as standards and used as such in all cases. With both the Nelson-Samuels method and the present method, there is close correlation between the "experimental" values and the "expected" values. Total 17-hydroxycorticosteroid levels are comparable by both methods. In addition the present method separates and estimates cortisol and 11-desoxycortisol independently even in quantities as small as 1 μg.

CLINICAL APPLICATION AND DISCUSSION

In recent years there has been increasing interest in the study of 11-desoxycortisol (substance S). Eberlein and Bongiovanni (10) found 11-desoxycortisol in the blood and its tetrahydro derivative in the urine of a patient with the hypertensive form of congenital adrenal hyperplasia. For the identification of 11-desoxycortisol in this patient's plasma they employed paper chromatography, utilizing the solvent system B5 of Bush (11). Touchstone *et al.* (12) found increased amounts of tetrahydro S in the urine of 3 patients with adrenal tumors, and Rosselet *et al.* (13) reported a similar finding in 2 patients with Cushing's syndrome. Bongiovanni *et al.* (14) studied the urinary metabolites of 11-desoxycortisol in normal children and adults, and suggested that the metabolism of 11-desoxycortisol differs in these different age groups. Using paper chromatography with a methylcyclohexane-dimethylformamide system, Touchstone *et al.* (15) found 11-desoxycortisol in human adrenal venous blood. Farrell and Lamus (16), using Burton's paper chromatographic method, found 11-desoxycortisol in the adrenal venous blood of dogs. Recently Gold (17) measured the "half-life" of 11-desoxycortisol by means of a modification (8) of the Peterson method (18), which measures total 17-hydroxycorticosteroids and does not separate cortisol from 11-desoxycortisol.

TABLE 4. SEPARATION OF CORTISOL AND 11-DESOXYCORTISOL FROM 10 ML. OF STORED BLOOD-BANK PLASMA AND COMPARISON TO NELSON-SAMUEL'S TOTAL 17-OH-CS METHOD
(See text for explanation)

Sample	Nelson-Samuels		Present method					
	Total 17-OH-CS (μg.)		Total 17-OH-CS (μg.)		Cortisol (μg.)		11-desoxycortisol (μg.)	
	Expected	Experimental	Expected	Experimental	Expected	Experimental	Expected	Experimental
Plasma		1.04		0.78		0.60		0.16
Plasma +3 μg. 11-desoxycortisol	5.14	4.40	4.15	3.75	0.60	0.49	3.16	2.90
Plasma +5 μg. cortisol	6.04	5.80	5.78	5.47	5.60	5.30	0.16	0.15
Plasma +3 μg. 11-desoxycortisol +5 μg. cortisol	10.14	8.85	9.15	8.85	5.60	5.40	3.16	3.06
Plasma		1.26		1.16		1.12		0.04
Plasma +1 μg. 11-desoxycortisol	2.33	2.57	2.22	2.10	1.12	0.85	1.04	1.18
Plasma +1 μg. cortisol	2.26	2.28	2.16	2.10	2.12	2.06	0.04	0.16
Plasma +1 μg. 11-desoxycortisol +1 μg. cortisol	3.33	3.40	3.22	3.32	2.12	2.00	1.04	1.24

SU-4885 test. Although amphenone B is known to inhibit steroidogenesis at the adrenal level, its toxic properties have limited its usefulness as an investigative tool. When Allen and Bencze (19) synthesized the compound 2-methyl-1,2-bis(3-pyridyl)-1-propanone, (SU-4885), Chart *et al.* (19) and Jenkins *et al.* (20) showed in dogs that this product selectively inhibited 11β-hydroxylation at the adrenal level. Thus, when SU-4885 is given to

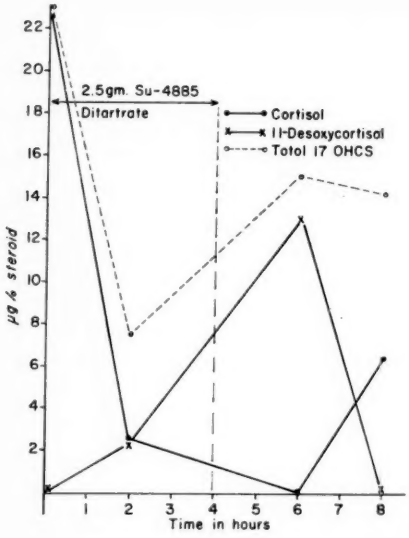

FIG. 3

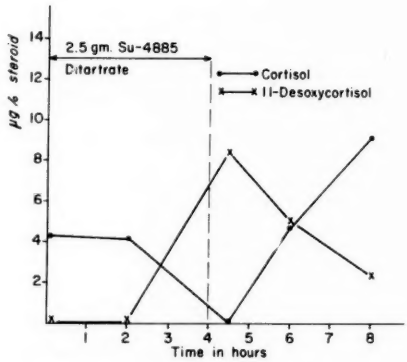

FIG. 4

FIGS. 3 and 4. Plasma steroid response to SU-4885 in 2 normal adults.

animals the production of cortisol is inhibited and (presumably through increased adrenal stimulation by endogenous corticotropin) 11-desoxycortisol is produced in excessive quantities. Desoxycorticosterone also is produced in increased quantities. Many investigators (21–27) now have utilized this phenomenon for studying steroidogenesis in man. In almost all cases the parameters they have investigated have been the levels of plasma total free 17-hydroxycorticosteroids or the urinary excretion of various metabolites of 17-hydroxycorticosteroids.

At present we are investigating various problems by means of the method reported here for estimation of cortisol and 11-desoxycortisol. When pituitary reserve (23) is tested by administration of SU-4885, measurements of cortisol and 11-desoxycortisol should be useful. Various parameters have been studied in this test, none of which specifically measures 11-desoxycortisol or cortisol. Figures 3 and 4 show the results of SU-4885 tests[4] on 2 normal adults. In each of these subjects there was no detectable 11-desoxycortisol, but a normal amount of cortisol in the control plasma sample. In both cases the administration of SU-4885 caused a decrease in the concentration of cortisol to undetectable levels, and a rise in the concentration of 11-desoxycortisol. When SU-4885 was discontinued, the 11-

[4] Metopirone® (SU-4885) kindly supplied by Dr. C. H. Sullivan, Ciba Pharmaceutical Products, Inc., Summit, N. J.

desoxycortisol level began to decrease while the cortisol level began to rise. Four hours after the administration of SU-4885 was terminated the plasma levels of both 11-desoxycortisol and cortisol approached control values. Figure 3 also shows the total 17-hydroxycorticosteroid levels in the same blood sample estimated by the Gold modification (8) of the Peterson method (18). The extra information obtained by measuring cortisol and 11-desoxycortisol is apparent.

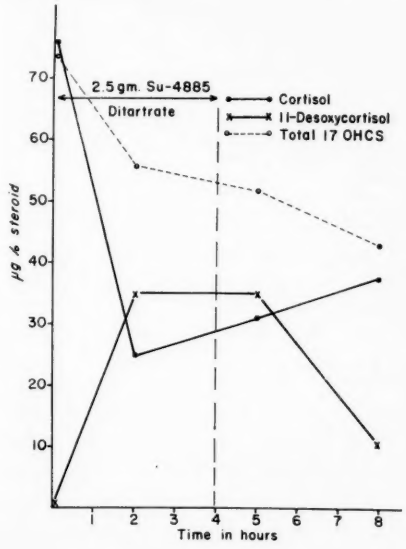

FIG. 5

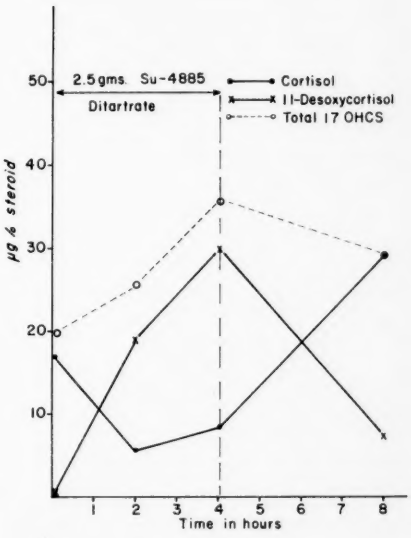

FIG. 6

FIGS. 5 and 6. Plasma steroid response to SU-4885 in 2 adults with adrenal hyperplasia. Note normal type of response but relatively high values.

Figures 5 and 6 show the results of similar tests on 2 patients with Cushing's syndrome due to adrenal hyperplasia. These patients responded to the administration of SU-4885, with a decrease in the plasma concentration of cortisol and an increase in that of 11-desoxycortisol. When SU-4885 was discontinued, the concentrations of the 2 steroids approached their control values. However, the absolute concentrations of both steroids in these patients were much greater than in normal subjects. Figures 5 and 6 also show the total 17-hydroxycorticosteroid levels (8) in these patients during the SU-4885 tests. The response to administration of SU-4885 in the patient whose data are shown in Figure 5 easily could be misinterpreted if only the total 17-hydroxycorticosteroid concentration were considered. There was no increase in the plasma total 17-hydroxycorticosteroid level

TABLE 5. EARLY MORNING PLASMA CORTISOL AND 11-DESOXYCORTISOL LEVELS IN NORMAL ADULTS

Subject	Cortisol (μg./100 ml.)	11-Desoxycortisol (μg./100 ml.)
C.M.	10.0	0
H.M.*	11.0	0
	6.3	0.8
	5.2	0.3
A.N.	12.7	0
E.R.	4.2	0
D.S.	22.6	0
L.L.	13.2	0
D.C.*	3.4	0.2
	3.5	0
M.H.*	2.1	0.1
	4.7	0.4
D.T.*	4.0	0
	2.6	0
P.M.*	3.3	0
	3.5	0
S.W.*	4.1	0.8
	4.4	0
E.B.*	12.8	0.8
	8.3	0.4
J.H.	10.7	0
P.F.	3.5	0
V.K.	7.8	1.4
B.Mc.	2.6	0
B.C.	11.8	0.8
W.C.	6.7	0

* Samples taken on different days.

when the SU-4885 was given, and thus no evidence of adrenal response to SU-4885. However, from the data regarding the concentrations of cortisol and 11-desoxycortisol, it is apparent that this patient did respond to SU-4885 administration by a decreased level of cortisol and an increased level of 11-desoxycortisol. In the patient whose data are shown in Figure 6 there was an increase in total 17-hydroxycorticosteroid concentration when the SU-4885 was given; however, as shown by the data regarding cortisol and 11-desoxycortisol concentrations, the pattern of response of these steroids to administration of SU-4885 was quite similar to that of the previous patient. Determination of both cortisol and 11-desoxycortisol indicates in both patients a normal, albeit exaggerated, response. Our findings in these 2 patients with adrenal hyperplasia correspond well with the findings of Jailer *et al.* (28), although different parameters were used to measure the response to SU-4885. As yet, we have not had an opportunity to study a patient with Cushing's syndrome due to adrenal carcinoma but, based on the work of Jailer *et al.* (28) in which they measured the urinary excretion of tetrahydro "S" after administration of SU-4885, we would expect an abnormal response in such a patient.

Touchstone *et al.* (16) found that less than one-tenth of the total steroids in human adrenal vein blood consisted of 11-desoxycortisol. Table 5 shows

the results of 26 determinations of cortisol and 11-desoxycortisol in the early morning blood samples of 18 normal adults. As shown in this table, values for 11-desoxycortisol in normal plasma vary from undetectable to minute quantities. In the cases in which 11-desoxycortisol was detected, it usually constituted less than one-tenth of the total measured 17-hydroxycorticosteroids, cortisol being the major steroid constituent.

The results presented indicate that the column chromatographic method described here for the measurement of cortisol and 11-desoxycortisol from a single blood sample is reliable. The additional information obtained by its use may prove of considerable value in supplementing the data obtained by other steroid technics. This method, either alone or in conjunction with the SU-4885 test, potentially can add much to the definitive evaluation of patients with adrenal or pituitary disorders.

Acknowledgment

The authors wish to express their gratitude to Dr. J. L. Bakke, Veterans Administration Hospital, Seattle, who kindly supplied the blood samples from patients with Cushing's syndrome and one of the normal subjects. The values for total 17-hydroxycorticosteroids by the Gold modification (8) of the Peterson method (18) were determined in his laboratory.

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THE EXCRETION OF PETTENKOFER CHROMOGENS BY HEALTHY SUBJECTS AND PATIENTS WITH VARIOUS ENDOCRINE DISORDERS*

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ABSTRACT

The earlier procedure by which dehydroepiandrosterone (DHA) was estimated on the basis of its digitonin precipitability and the Zimmermann reaction is of limited significance, because the 2 artifacts (Δ^5-3β-chloroandrosterone-17 and Δ^{3-5}-androstadienone-17) that are produced from DHA during hot-acid hydrolysis are not precipitable by digitonin. Among the different analytical procedures for estimating DHA, one that is commonly applied is the method of Munson *et al.*, in which the Pettenkofer reaction is performed for the determination of DHA and its 2 chief artifacts. In the present study, the Zimmermann and Pettenkofer reactions were performed on the ketonic alpha and beta fractions of extracts obtained from the urine of healthy persons, patients with various endocrine disorders, and after administration of ACTH to patients with adrenocortical insufficiency. 17-Hydroxycorticosteroids were assayed in all cases as a control for adrenal activity. Determination of Pettenkofer chromogens in the urinary ketonic extracts after hot-acid hydrolysis yielded a mean value for normal subjects that represented 31 ± 4.5 per cent of the total 17-ketosteroid (17-KS) output in both males and females. The mean values for Pettenkofer chromogens in various endocrine disorders ranged from 0.15 mg. to 9.72 mg. per twenty-four hours, representing from 8 to 98 per cent of the total 17-KS excretion. The sum of the Pettenkofer chromogens in the alpha plus beta fraction after digitonin precipitation averaged 81 per cent of the value for the unfractionated extract. Recovery of Zimmermann chromogens measured in the same alpha plus beta fraction was 94 per cent of the value for the unfractionated extract. Thus there is unspecific Pettenkofer chromogenic material in the urinary ketonic fractions after hot-acid hydrolysis. This may, in part, account for the high values commonly obtained for urinary DHA when the Pettenkofer reaction was performed with those extracts. Although the excretion of Pettenkofer chromogens is considered to reflect adrenal activity and may have clinical significance, values for these chromogens in the neutral ketonic fraction after hot-acid hydrolysis must not be taken as the urinary output of DHA.

ANDROSTERONE, etiocholanolone and dehydroepiandrosterone (DHA) are the three most abundant urinary 17-ketosteroids (17-KS). Urinary androsterone and etiocholanolone are derived from more than

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one precursor and variation in the urinary excretion may reflect very different processes.

DHA originates only in the adrenals; therefore the amount in the urine directly reflects adrenal activity. The sulfate is the principal conjugate of DHA in plasma and in urine, but the extreme sensitivity of the steroid moiety of the sulfate during acid hydrolysis at elevated temperature frequently results in underestimation of the urinary level.

When chromatographic methods have been applied (1, 2), satisfactory resolution of the urinary 17-ketosteroids has been achieved. The values for DHA obtained by these methods, however, have been considerably greater than those reported by earlier workers when DHA was measured as its precipitate with digitonin. This was so because, after the hot-acid treatment, the two artifacts from DHA (Δ^5-3β-chloroandrosterone and Δ^{3-5}-androstadien-17-one) are not precipitable with digitonin, thus reducing the final value for DHA. Also, in addition to the low results obtained for beta 17-KS when this method is applied, the technique for precipitating the digitonides requires too much time and is too difficult to be used with good results as a routine method for clinical application. Chromatographic methods for fractionation of 17-KS are also too laborious for routine use. Another method for the determination of DHA is that of Munson *et al.* (3). Values for DHA obtained by this procedure are higher than those obtained by the methods previously mentioned; nevertheless, the method is frequently applied in clinical studies, and in some textbooks (4–6) is recommended for the determination of DHA in urine.

It is the purpose of this paper to present a comparison of the values for Pettenkofer chromogens as determined by the method of Munson *et al.* in the urine of healthy subjects and patients with various endocrine disorders, and the values obtained when DHA was estimated as the digitonin-precipitated fraction of the 17-ketosteroids, determined in both alpha and beta fractions by the Zimmermann and the Pettenkofer reactions.

As a control for the state of adrenal activity, urinary 17-hydroxycorticoids (Porter-Silber chromogens) were also assayed in all subjects studied.

METHODS

Urine was collected in 24-hour specimens. A small aliquot of urine was taken for the 17-hydroxycorticosteroid (17-OH-CS) determination. The rest of the 24-hour specimen was boiled with 15 volumes per cent of concentrated hydrochloric acid for eight minutes to hydrolyze the steroids. The resulting mixture was cooled under running water and subjected to extraction with ether. The extracts were washed with 2*N* sodium hydroxide solution and water. The ketonic fraction was separated by means of Girard reagent (7), and the 17-ketosteroid (17-KS) content was determined by the Zimmermann reaction (8). The Pettenkofer chromogens (P.C.) were also determined in the ketonic fraction (3). The remainder of the extract was divided into two fractions: (*a*) not precipitable by

digitonin (or alpha), and (*b*) precipitable by digitonin (or beta), according to the method of Butt *et al.* (9). Zimmermann and Pettenkofer chromogens were measured in both alpha and beta fractions.

The method of Munson *et al.* (3) was used for the determination of P.C., except that 10 ml. of 16*N* sulfuric acid was added instead of 7.5 ml. The color intensity was determined in a Hilger Sppeker (H760) spectrophotometer with filter No. 1. Standards of 20 and 40 μg. of DHA were run with each determination. Readings for urine extracts with optical densities higher than those obtained for the standards were discarded, and the determination repeated with a smaller aliquot.

17-OH-CS determinations were performed by a modification (10) of the Silber-Porter method (11).

TABLE 1. URINARY 17-HYDROXYCORTICOSTEROIDS, 17-KETOSTEROIDS, AND PETTENKOFER CHROMOGENS (P.C.) IN 27 NORMAL SUBJECTS—MEAN VALUES WITH STANDARD DEVIATIONS

Sex	No. of determinations	17-OH-CS* (mg./24 hrs.)	17-KS** (mg./24 hrs.)	P.C. (mg./24 hrs.)	P.C. (% of tot. 17-KS)
Female†	15	5.81 (±2.14)	6.14 (±1.60)	1.96 (±0.70)	31 (±4.4)
Male‡	12	9.39 (±1.47)	12.12 (±3.50)	3.61 (±1.17)	31 (±4.6)

* Porter-Silber.
** Ketonic fraction.
† Age range, 20–43 years.
‡ Age range, 18–45 years.

RESULTS

Normal men and women

In the urine of 27 normal subjects, DHA was determined as Pettenkofer chromogens (P.C.) in the ketonic fraction after hot-acid hydrolysis. The values are listed in Table 1. The mean values for P.C. were 3.61 ±1.17 (S.D.) mg. per twenty-four hours in males and 1.96 ±0.70 mg. in females. These amounts represented 31 ±4.5 per cent of the total 17-KS output in both normal males and females.

The mean 24-hour value for urinary 17-KS in males was 12.12 ±3.50 mg. and in females 6.14 ±1.60 mg. For urinary 17-OH-CS, the mean values were 9.39 ±1.47 mg. in males and 5.81 ±2.14 mg. in females.

Adrenal insufficiency; ACTH test

Urines of 5 patients with various abnormalities of adrenocortical function were assayed.[1] The results are listed in Table 2.

The average excretion of P.C. in the urine of the 4 patients with *Addison's disease* was 0.58 mg. per twenty-four hours, representing 17 per cent

[1] All the clinical subjects studied in this paper were seen in the Instituto de Patología Médica del Hospital Provincial de Madrid, the Director of the Institute and Professor of Endocrinology being the late Dr. G. Marañón.

TABLE 2. RESULTS OF ACTH TEST (8-HR., I.V.) ON 17-HYDROXYCORTICOSTEROIDS, 17-KETOSTEROIDS AND PETTENKOFER CHROMOGENS (MG./24 HRS. ±S.D.) IN PATIENTS WITH ADRENAL INSUFFICIENCY

Subjects	Time after ACTH (hrs.)	17-OH-CS	17-KS	P.C.	P.C. (% of tot. 17-KS)
Addison's disease (4 males; age range 22–42 yrs.)	0–24	2.42 (±0.75)	3.21 (±1.19)	0.58 (±0.14)	17 (±3.83)
	24–48	2.30 (±1.28)	3.84 (±0.63)	0.70 (±0.20)	19 (±5.74)
		2.92 (±0.99)	3.25 (±1.04)	0.61 (±0.10)	17 (±3.05)
Adrenal insufficiency with acromegaly (M.L., female aged 34)	0–24	1.98	0.78	0.28	36
	24–48	1.30	0.65	0.30	46
	48–76	5.48	0.90	0.33	37
		2.54	0.92	0.33	36
Controls (1 F & 2 M hospital patients with normal adrenal function)	0–24	6.03	7.09	2.76	37
	24–48	15.76	11.20	5.37	49
		5.87	4.93	2.21	38

of the total 17-KS. Stimulation with corticotropin (25 mg. of ACTH administered intravenously over a period of eight hours) had no effect on 17-KS excretion (Table 2).

In Patient M.L. with adrenal insufficiency (Table 2), amenorrhea had followed delivery of her second child. Two years afterward, when she was 28 years old, she noticed that she needed larger shoes; later she observed that her hands were larger, her face deformed and her skin thickened. Her voice changed, and hearing became poor. She had headaches and was weak, tired and depressed. There was no mucosal pigmentation, and no clinical evidence of enlargement of the thyroid, nor was there any adenopathy. Steroid excretion was well below normal levels. The clinical diagnosis in this case was *adrenocortical insufficiency* and acromegaly. The eight-hour ACTH test evoked a slight response in the urinary corticosteroid levels and also in the percentage of Pettenkofer chromogens (Table 2).

Three hospitalized subjects (1 female and 2 males) with apparently normal adrenal function were used as *controls* in the ACTH test. As shown in Table 2, the corticosteroid output increased approximately threefold above the basal levels within twenty-four hours after the beginning of the eight-hour ACTH infusion. Both the 17-KS and the P.C. increased to about twice the control values. For these subjects, as for the addisonian patients, the basal values represented the average of at least two determinations on urines collected one or two days before starting the administration of ACTH.

Various endocrinopathies

Levels of P.C. excretion in various types of endocrine disorders (Tables 3 and 4) ranged from 0.15 mg. to 9.72 mg. per twenty-four hours. These

values represented from 14 to 98 per cent of the total urinary 17-KS excretion. Some of these findings were of clinical significance.

In Patient M.M. (Table 3) the clinical diagnosis was *pseudohermaphroditism* due to congenital adrenal hyperplasia. When this girl was 6 months old she had a greatly enlarged clitoris; at the age of 6 years she had excessive pubic hair of the masculine type and a peniform clitoris. The ovaries were normal histologically (biopsy). The extremely high excretion of 17-KS (Table 3) persisted after an operation involving removal of one adrenal weighing 25 Gm. and the peniform clitoris measuring 4 cm. in length. 17-OH-CS excretion was slightly lower after, than before the operation. P.C.

TABLE 3. EXCRETION OF 17-HYDROXYCORTICOSTEROIDS, 17-KETOSTEROIDS AND PETTENKOFER CHROMOGENS (MG./24 HRS.) IN 3 PATIENTS WITH ADRENAL DYSFUNCTION, BEFORE AND AFTER UNILATERAL ADRENALECTOMY*

Subject and diagnosis	Sex & age	Time after adrenalectomy (wks.)	17-OH-CS	17-KS	P.C.	P.C. (% of tot. 17-KS)
M.M.						
Pseudohermaphrodism	F6	—	8.10	36.90	9.72	26
		2	6.18	29.45	7.86	27
		6	5.33	40.30	6.87	17
J.S.						
Virilism	F19	—	20.96	24.01	3.92	16
		20	6.83	10.50	3.26	31
C.L.						
Cushing's adenoma	F24	—	23.20	6.58	1.26	19
		—	32.10	7.94	1.67	21
		24	2.00	1.44	0.77	53
		48	3.70	1.14	0.52	47

* See text.

excretion was 26 per cent of the total 17-KS excretion before operation; it diminished to 17 per cent postoperatively.

Two cases of *virilism* were studied (Table 4). Patient J.S. was a 19-year-old woman who first noticed an abnormal growth of hair on the arms and legs at the age of 14 years. At that time her weight was 80 Kg. and the pubic hair pattern was masculine. Later the following symptoms were observed: abnormalities of the menstrual cycle, a deepening of the voice, a transition of body contour from the feminine to the masculine type, and enlargement of the clitoris. Excretion of 17-OH-CS and 17-KS was high, and the proportion of P.C. (16 per cent) was low. The sex chromatin pattern was positive (43 per cent Barr bodies in the nuclei of the buccal epithelial cells). One adrenal containing a polynodular adenoma (13 Gm.) was removed from this patient. Five months after the operation the excretion of 17-OH-CS and 17-KS was in the normal range and the proportion of P.C. rose to the normal value of 31 per cent of the total 17-KS excretion (Table 3). The other patient with virilism (R.V., Table 4) was a 28-year-old wom-

TABLE 4. EXCRETION OF 17-HYDROXYCORTICOSTEROIDS, 17-KETOSTEROIDS AND PETTENKOFER CHROMOGENS (MG./24 HRS.) IN PATIENTS WITH MISCELLANEOUS ENDOCRINOPATHIES OR HORMONE-TREATED DISORDERS

Name, age sex	Diagnosis	17-OH-CS	17-KS	P.C.	P.C. (% of tot. 17-KS)
B.F. —23 F	Cushing's syn.	9.70	16.04	4.55	28
T.M. —32 F	Cushing's syn.	15.77	21.50	7.53	35
C.L.* —24 F	Cushing's syn.	27.65	7.26	1.47	20
A.M. —11 M	Precocious puberty; macrogenitosomia	5.50	3.16	3.10	98
F.V. —11 M	Precocious puberty	6.82	6.16	1.93	31
M.B. —13 M	Fröhlich's syn. (adiposogen. dystrophy)	2.98	2.76	0.39	14
M.M. — 6 F	Pseudohermaphrodism	8.10	36.90	9.72	26
J.S.** —19 F	Virilism	20.96	24.01	3.92	16
R.V. —28 F	Virilism	2.72	3.68	1.93	52
P.P. —15 F	Cortical hyperfunction	16.52	17.44	3.32	19
A.I. —27 M	Cortical hyperfunction	15.28	21.50	6.83	32
P.G. —21 F	Hirsutism	7.64	9.48	2.84	30
D.N. —17 F	Hirsutism	5.42	8.96	1.91	21
C.D. —17 F	Hirsutism	6.47	11.55	2.82	24
G.F. —30 F	Hirsutism	7.31	11.43	3.16	28
J.Y. —30 F	Hirsutism; diabetes	9.97	7.25	3.12	43
C.A. —12 F	Simmonds' disease	3.28	1.44	0.24	17
C.G. —43 F	Sheehan's syndrome	0.37	1.10	0.47	43
M.S. — 7 M	Hypothyroidism	2.60	1.87	0.15	79
M.P. —15 M	Rheumatic endocarditis	3.96	3.68	1.00	27
	(corticoid therapy)	10.85	5.54	5.46	98
M.M. —47 F	Carcinomatosis; origin in breast† (20 days later)	3.85	14.90	1.19	8
		5.47	15.58	1.22	8

* See Table 3; average of 2 determinations.
** See Table 3.
† This patient was being treated with testosterone.

an who had never menstruated ("primary amenorrhea"). Axillary hair was lacking until the age of 20. Six years later, fatty breasts began to develop. The uterus was very small. On pelvic examination the clitoris appeared of normal size. The patient was belligerent and lacked normal moral sense. The sex chromatin pattern was negative (3.6 per cent Barr bodies in the nuclei of the buccal epithelial cells and 2 per cent in leukocytes). The excretion of 17-OH-CS and 17-KS was low, and the proportion of P.C. in relation to total 17-KS was fairly high (52 per cent). The difference between the clinical pictures in these 2 patients was reflected in the steroid excretions.

Steroid excretion data on 3 female patients with *Cushing's syndrome* are presented on Table 4. All 3 exhibited the characteristic signs, *viz*, moon-shaped face, purple striae, truncal obesity, prominent abdomen, thick neck, slender extremities, and a steroid excretion above normal levels. After two months of steroid therapy in Patient T.M., menstruation began and general improvement was observed. The disease was more severe in Patient C.L., from whom an adrenal adenoma was removed that weighed

11.5 Gm. After the operation the daily excretion of 17-OH-CS decreased tenfold, that of 17-KS sixfold, and that of P.C. nearly threefold. The proportion of P.C. was 20 per cent of the total 17-KS before adrenalectomy, and 50 per cent afterwards. These values were practically unchanged about a year after the removal of the tumor.

In the 5 women with *hirsutism* there was a generalized increase of hair growth, with a male pattern of distribution. Body habitus and external genitalia were normal. Steroid excretion was fairly normal. Patient J.Y. also had diabetes; in the urine the proportion of P.C. was 43 per cent of the total 17-KS, *i.e.*, slightly above the percentage for the normal females

TABLE 5. ZIMMERMANN AND PETTENKOFER REACTIONS IN THE KETONIC ALPHA AND BETA FRACTIONS OF URINE FROM NORMAL SUBJECTS AND THOSE WITH VARIOUS ENDOCRINE DISORDERS

Diagnosis	No. of cases	17-Ketosteroid fractions (mg./24 hrs.)			Pettenkofer chromogen fractions (mg./24 hrs.)		
		K	alpha	beta	K	alpha	beta
Normal males	6	10.72	9.83	0.28	3.47	2.43	0.24
Normal females	6	6.04	5.45	0.16	1.95	1.40	0.22
Cushing's syn.*	6	10.62	9.98	0.08	3.12	2.45	0.02
Adrenal hypofunction†	2	1.63	1.45	0.01	0.34	0.30	0.00
(after ACTH)	[6]	1.64	1.42	0.01	0.42	0.31	0.00
Pseudohermaphrodism	1	36.90	33.45	0.13	9.72	9.00	0.05
Virilism	1	3.68	2.95	0.48	1.93	1.05	0.51
Precocious puberty; macrogenitosomia.	1	3.16	2.86	0.02	3.10	2.08	0.00
Fröhlich syn.; (adiposogen. dystrophy)	1	2.76	2.20	0.24	0.40	0.36	0.04
Hirsutism	3	9.46	8.74	0.10	2.26	1.69	0.03

* Four females and 2 boys aged 10 and 11 years.

† One patient with adrenal insufficiency, and 1 with Addison's disease, before and after an ACTH test; see text.

studied by us, and the highest percentage for the group of patients with hirsutism.

Two boys with *precocious puberty* were studied. Patient A.M. exhibited macrogenitosomia; Patient F.V. did not. The excretion of 17-OH-CS and 17-KS was in the normal range for both boys. However, the proportion of P.C. was 98 per cent of the total 17-KS in Patient A.M., whereas it was 31 per cent (normal value) in Patient F.V. (Table 4).

In a boy (M.B.) with *Fröhlich's syndrome* (adiposogenital dystrophy), 17-OH-CS and 17-KS excretion was low, and the proportion of P.C. (14 per cent) was also low (Table 4).

In the case of *rheumatic endocarditis*, a dramatic rise in the proportion of P.C., from 27 to 98 per cent, was observed while the patient (M.P.) was being treated with the corticoids, Dacortin and Protandren. 17-OH-CS ex-

cretion was increased about twofold. The interval between the pre-treatment and post-treatment determinations was twenty days.

Patient M.M. had *carcinomatosis*, of mammary origin. She was receiving testosterone propionate. Determinations were made at twenty-day intervals. The high excretion of 17-KS and the low proportion of P.C. (8 per cent) were to be expected in view of the products of testosterone and DHA metabolism. The fact that the proportion of P.C. was the same in the second determination is further confirmation of the validity of the methods employed.

Zimmermann versus Pettenkofer chromogens (all subjects)

When the Zimmermann and Pettenkofer reactions were performed in the ketonic alpha and beta fractions (Zimmermann chromogen reaction) the

TABLE 6. RECOVERY OF TOTAL ZIMMERMANN AND PETTENKOFER CHROMOGENS AFTER DIGITONIN SEPARATION

Diagnosis	No. of determinations	Zimmermann reaction (% ±S.D.)	Pettenkofer reaction (% ±S.D.)
Normal males	6	94 (±7.7)	77 (±12.4)
Normal females	6	93 (±4.9)	85 (± 5.4)
Cushing's syn.	6	95 (±6.5)	79 (±11.8)
Addison's dis.	8	88 (±7.6)	81 (±14.8)
Various endocrine disorders	7	91 (±1.9)	83 (±12.2)

resulting values represented 3 per cent of the total 17-KS (Table 5). The highest proportions found were 10 per cent in 1 male and 11 per cent in 1 female. These results are in agreement with those of most authors.

In normal subjects the sum of the Pettenkofer chromogens in the alpha and beta fractions after digitonin precipitation (Table 6) was nearly always lower (an average of 81 per cent) than the value obtained for the unfractionated extract. However, the sum of the Zimmermann chromogens in the same extracts was 94 per cent of the value for the unfractionated extract. In 6 patients with Cushing's syndrome, a similar 20 per cent of the Pettenkofer chromogens was lost on precipitation with digitonin. Similar results were obtained in several cases of other endocrine disorders, and in the 2 cases of adrenal hypofunction after administration of ACTH.

DISCUSSION

The values for excretion of corticosteroids and ketosteroids in healthy subjects as reported in this paper are in agreement with those found by most investigators who have applied the same methods. However, our values for the excretion of Pettenkofer chromogens do not correspond to

those of other investigators. Because of the extremely high readings for the blanks, the determination of P.C. in crude neutral urinary extracts after hot-acid hydrolysis is useless, and the ketonic fraction must be used for assay.

In contrast to the variation in P.C. excretion between subjects, the excretion was remarkably constant in a given individual, whether normal or diseased. The proportion of the urinary total 17-KS measured by the Pettenkofer reaction in the normal persons studied was 31 ±4.5 per cent. These results do not quite agree with those of Landau *et al.* (12), whose values ranged from 10 to 63 per cent of the 17-KS. Our data agree more with those of Bray and Merivale (13), who found that DHA constituted as much as 50 per cent of the total 17-KS, though most commonly between 25 and 40 per cent. However, the technical procedures used by these authors were somewhat different from ours.

The values for the digitonin-precipitable fraction by the Zimmermann reaction averaged 3 per cent. This percentage is in agreement with that found by other investigators. Dobriner *et al.* (14) concluded that the beta fraction was from 0.6 to 2.2 per cent of the total 17-KS; Pincus (15) found that it rarely exceeded 15 per cent and was usually 2 to 3 per cent; Butt *et al.* (9) reported it to be less than 10 per cent; Dorfman and Shipley (4), from 0 to 18 per cent; and Loraine (16), less than 15 per cent.

When the ketonic fractions were separated into alpha and beta steroids, our results indicated that a portion of Pettenkofer-reacting material was lost in the process of separation. If the Pettenkofer reaction were specific for only the ketonic steroid identified as chromogens by Munson *et al.* (dehydroepiandrosterone and its 2 chief hot-acid hydrolysis artifacts, Δ^5-3β-chloroandrosterone and $\Delta^{3,5}$-androstadien-17-one), the sum of the alpha and beta fractions in terms of the percentage found in the ketonic fraction should be the same in the Zimmermann and Pettenkofer reactions. However, we obtained average recoveries of 96 per cent for the Zimmermann chromogens and only 80 per cent for the Pettenkofer chromogens.

Since DHA and its 2 principal transformation products yield a colored product with the Pettenkofer reaction, the losses that were observed after the digitonin precipitation ought to have been due to nonspecific chromogenic material present in the urinary ketonic extracts after hot-acid hydrolysis. The loss of reacting compounds was of variable degree. A study of the Pettenkofer-reacting material in urinary extracts obtained after different methods of hydrolysis is in progress in our laboratory. From our results so far we may conclude that the Pettenkofer chromogenic material that is assayed in urinary ketonic extracts after hot-acid hydrolysis is an index of adrenal activity, although the values may not represent the urinary excretion of DHA. Therefore the method of Munson *et al.* should not be used for

the estimation of DHA. More work would be desirable in some of the endocrine disorders we have studied to see whether the Pettenkofer reaction can be of value as an additional index of steroid metabolism.

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VALUE OF DRUMSTICKS AND OTHER NUCLEAR APPENDICES IN THE DETERMINATION OF SEX. DISCRIMINATORY ANALYSIS BASED ON FINDINGS IN 804 NORMAL SUBJECTS

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ABSTRACT

In order to find a better way to interpret findings based on examination of drumsticks and other nuclear appendices in polymorphonuclear neutrophils, a study was made of 804 normal subjects of phenotypically well-defined sex and divided into equal groups of 402 men and 402 women. The following nuclear appendices were studied and accurately defined: a) drumstick; b) sessile nodule; c) small club; d) balloon. After submission of the data to adequate treatment in order to normalize the distribution, a discriminant function was established: $Y_2 = Y_1 + 0.3(Y_3 - Y_4)$. From this analysis it was concluded that the criterion of Davidson and Smith is correct, *i.e.*, chromatin-positive (female) subjects are those with 6 or more drumsticks per 500 polymorphonuclear neutrophils. However, 28.0 per cent of the women showed less than 5 drumsticks; and 9.7 per cent of the men showed 1 or more drumsticks per 500 p.leukocytes. By adopting a discriminant function including drumsticks, sessile nodules and small clubs, it was possible to classify the majority of these cases according to their true sex, with an error of about 0.7 per cent. On the basis of these data a nomogram was prepared, which permits a direct reading that is simple and sufficiently accurate.

THE sex chromatin described by Barr *et al.* in 1950 (1) represented a new approach to an understanding of the problems of sex. Work by groups such as those of Ford (2, 3) and Lejeune (4) has provided a new fund of information and defined more precisely the significance of sex chromatin.

In view of the delicacy of technique and interpretation required for chromosomal methods, the determination of sex on the basis of sex chromatin is a comparatively simple procedure. It is therefore invaluable for use in medical practice.

Determination of the sex chromatin in polymorphonuclear neutrophils (PMN), as described by Davidson and Smith (5), is one of the methods that is considered to be sufficiently accurate for this purpose (6–12). Defini-

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tions of the nuclear appendices of the PMN, however, are not uniform (13), so that comparison of the findings is difficult. On the other hand, discrepancies have been reported, not only between the sex chromatin of the PMN and the true sex of the subject (14), but also between the sex chromatin of the PMN and that of cells from smears of the buccal mucosa (15, 16).

The purpose of the present work was to re-examine the data and work out a better way of interpreting the findings supplied by examination of the drumsticks and other appendices in the nuclei of PMN. We therefore carried out a statistical analysis for the purpose of establishing, with minimum risk of error, the sex of the subject. No attempt was made to correlate the findings with those obtained by the buccal smear method.

METHODS

A study was made of 804 subjects (402 of each sex), all considered to possess completely defined phenotypical sex characteristics.

A sample of blood was taken from each subject and used to prepare a plate by the technique of enriched smears (17).

A count was made on each plate of the nuclear appendices found in 500 PMN—the cells with two or more lobules being selected. Identity of the material was not known in advance. This work, which was carried out by two of the authors, was preceded by a period of training intended to establish uniform criteria of classification for the appendices being studied.

The following definitions were adopted:

Drumstick (Fig. 1, c and d)—An appendix characterized by its round or drop-like form, attached to one of the lobules by a thin stem of chromatin. It has a diameter of approximately 1.5 micra, and colors intensely. The form is constant. Not more than 1 is found in the same cell. Drumsticks generally exist in mature neutrophils (more than two lobules).

Sessile nodule (Fig. 1, a)—A formation protruding from a lobule like a bud. It has the same diameter and coloration characteristics as a drumstick.

Small club (Fig. 1, e and f)—This term is applied only to an appendix similar in shape to the drumstick, but generally smaller and only slightly basophilic.

Balloon (Fig. 1, b)—Spherical in form; the chromatin is well condensed, touching the nucleus only lightly and without a visible stem. This appears to be a transition form between the sessile nodule and the drumstick (8, 18).

RESULTS AND COMMENTS

Results are presented in Table 1. They are not percentual, but are based on a count of the appendices found in 500 neutrophils for each subject. The distribution of the 804 subjects is shown for each sex, according to the number of nuclear appendices of each type. For instance, there were 9 subjects of the female sex with 0 drumsticks, 7 subjects with 1 drumstick, and so on.

With the exception of the distribution corresponding to the balloon-type nuclear appendix, of male sex phenotype, the others showed variance (to the level of 1 per cent) significantly greater than the respective averages

(over dispersion), in variable degree approximating negative binomial distribution (19). These distributions were reasonably normalized, the number of nuclear appendices being expressed under the form (20):

$$y_p = k^{-1} \text{ arc. sine } [k(x_p + \tfrac{1}{2})^{\frac{1}{2}}]$$

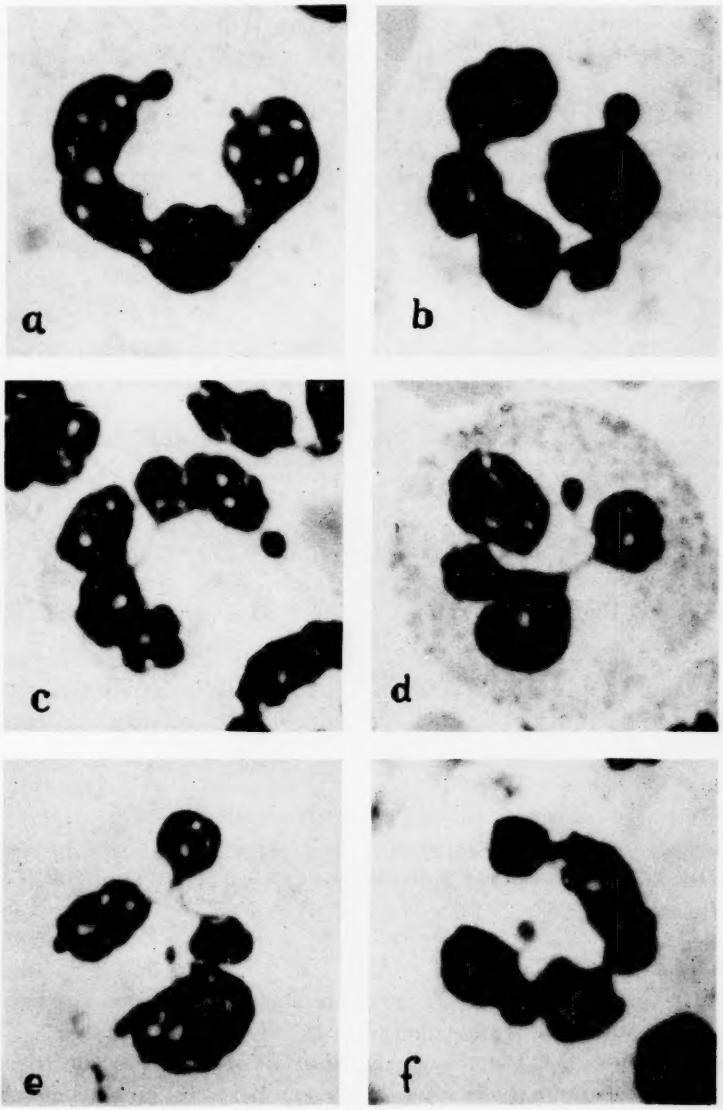

FIG. 1. a) sessile nodule; b) balloon; c) drumstick; d) drumstick; e) and f) small clubs.

TABLE 1. FREQUENCY DISTRIBUTION OF THE 804 SUBJECTS, ACCORDING TO SEX AND NUMBER OF NUCLEAR APPENDICES OF EACH TYPE*

No. of nuclear appendices x_p	Female sex				Male sex			
	Drumstick p=1	Balloon p=2	Sessile nodule p=3	Small club p=4	Drumstick p=1	Balloon p=2	Sessile nodule p=3	Small club p=4
0	9	88	81	50	363	398	384	30
1	7	112	90	50	31	4	10	16
2	7	93	99	74	4	0	4	26
3	23	61	54	59	2	0	2	41
4	24	32	33	42	2	0	1	37
5	43	10	25	37	0	0	0	44
6	53	3	14	25	0	0	1	35
7	36	1	3	20	0	0	0	28
8	31	1	1	13	0	0	0	19
9	20	0	0	10	0	0	0	12
10	36	0	2	4	0	0	0	23
11	27	1	0	5	0	0	0	14
12	16	0	0	2	0	0	0	8
13	10	0	0	2	0	0	0	4
14	12	0	0	3	0	0	0	6
15	9	0	0	3	0	0	0	8
16	6	0	0	0	0	0	0	0
17	7	0	0	0	0	0	0	4
18	5	0	0	1	0	0	0	5
19	7	0	0	0	0	0	0	5
20 or more	14	0	0	2	0	0	0	37
Total	402	402	402	402	402	402	402	402
Average ($\bar{x}_p$)	$\bar{x}_1=8.63$	$\bar{x}_2=1.75$	$\bar{x}_3=2.09$	$\bar{x}_4=3.76$	$\bar{x}_1=0.13$	$\bar{x}_2=0.01$	$\bar{x}_3=0.08$	$\bar{x}_4=7.78$
Variance (S_p^2)	$S_1^2=27.63$	$S_2^2=2.26$	$S_3^2=3.20$	$S_4^2=11.81$	$S_1^2=0.22$	$S_2^2=0.0099$	$S_3^2=0.23$	$S_4^2=50.10$
Standard (S_p) deviation	$S_1=5.3$	$S_2=1.5$	$S_3=1.8$	$S_4=3.4$	$S_1=0.5$	$S_2=0.099$	$S_3=0.5$	$S_4=7.1$

* The index, p, indicates the type of nuclear appendix (p=1 for drumstick, 2 for balloon, 3 for sessile nodule and 4 for small club); x_p is the number of nuclear appendices of type p; and $\bar{x}_p$, S_p^2 and S_p are respectively the average, variance and the standard deviation of x_p.

in which p is the type of nuclear appendix (p = 1 for drumstick, 2 for balloon, 3 for sessile nodule and 4 for small club), x_p is the number of nuclear appendices of the type p, and k is a constant which we estimated as follows (21):

$$k = \sqrt{\frac{\sum_{i=1}^{4} S_p^2 - \sum_{p=1}^{4} \bar{x}_p}{\sum_{i=1}^{4} \bar{x}_p^2}}$$

in which $\bar{x}_p$ and S_p^2 are, respectively, the average and the variance of x_p.

On the basis of the transformed values (y_p) we established the discriminant function (22, 23):

$$Y_1 = y_1 + 0.094y_2 + 0.315y_3 - 0.274y_4$$

in which y_1, y_2, y_3 and y_4 are, respectively, the number of drumsticks, balloons, sessile nodules and small clubs in transformed units.

This function, the theoretical distribution of which for both sexes is represented in Figure 2, shows a highly significant difference between the sexes, as may be seen in Table 2.

The discriminant function proposed, therefore, permits a good differentiation between the sexes. If we adopt as the point of separation between them the point $P_{Y_1} = 1.333$, equidistant between the average of the values of Y_1 for the female sex ($Y_{1F} = 2.167$) and the average of the values of Y_1 for the male sex ($\bar{Y}_{1M} = 0.499$), this would classify in the male sex all those subjects showing a value of Y_1 less than P_{Y_1}, and in the female sex all the rest of the subjects. Thus we would classify erroneously those subjects of the male sex with a value of Y_1 equal to or greater than P_{Y_1} and those subjects of the female sex with value of Y_1 less than P_{Y_1}. This would include all subjects with a value of Y_1 that departs from the average corresponding to the sex to which it belongs, and in the direction of the average corresponding to the other sex, by a value equal to or greater than $1.333 - 0.499$ (or $2.167 - 1.333$) $= 0.834$. The intra-sex standard deviation is $S_{Y_1} = \sqrt{0.115} = 0.339$; therefore, a departure equal to 0.834 or $0.834/0.339 = 2.46$ in units of standard deviation will be exceeded unilaterally, by chance in 0.7 per cent of the cases.

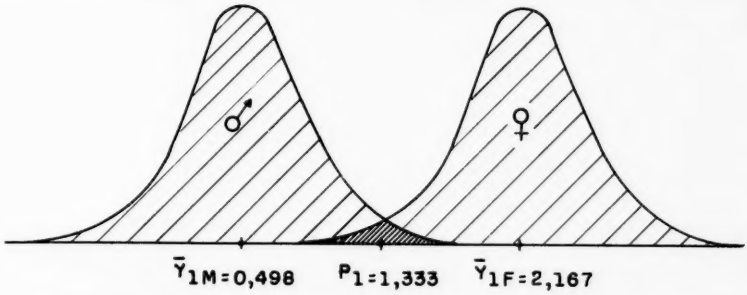

FIG. 2. Theoretical distribution of discriminant function for both sexes.

Thus if we adopt the discriminant function proposed and be guided by this criterion, it is to be expected that about 0.7 per cent of the subjects will be incorrectly classified. We say "about" 0.7 per cent, because the transformation adopted is only approximate and the normalization obtained is thus not perfect, especially when we approach the point of separation P_{Y_1}.

The discriminant function proposed is, nevertheless, susceptible of simplification. In effect, if in the function Y_1 we make the coefficient of y_1 (balloon) equal to zero we have:

$$Y_2 = y_1 + 0.315y_3 - 0.274y_4$$

or approximately

$$Y_2 = y_1 + 0.3(y_3 - y_4).$$

Corresponding to this new function are the averages $\overline{Y}_{2F} = 1.992$ and $\overline{Y}_{2M} = 0.374$, and the point of separation $P_{Y_2} = 1.183$. The intra-sex standard deviation being $S_{Y_2} = 0.330$, a departure equal to or greater than $1.183 - 0.374 = 0.809$ (or $1.992 - 1.183 = 0.809$), or 2.45 in units of standard deviation, will occur by chance in 0.71 per cent of the cases—practically 0.7 per cent.

As may be seen, this function is practically as good as the former, since the contribution of the appendix eliminated (balloon) for classification of sex is insignificant.

Application of the foregoing function, however, is not direct. The values y_1, y_3 and y_4 represent, respectively, the number of drumsticks, sessile nodules and small clubs in transformed units.

TABLE 2. ANALYSIS OF VARIANCE OF Y_1

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between sexes	559.789	4	139.947	1216.9
Within sexes	91.799	799	0.115	
Total	651.588	803		

The same function in original units would be:

$$X_2 = \frac{\left[k\sqrt{x_1 + \frac{1}{2}} + \sqrt{k^2(x_1 + \frac{1}{2}) + 1}\right]^{1/0.3}\left[k\sqrt{x_3 + \frac{1}{2}} + \sqrt{k^2(x_3 + \frac{1}{2}) + 1}\right]}{k\sqrt{x_1 + \frac{1}{2}} + \sqrt{k^2(x_4 + \frac{1}{2}) + 1}}$$

in which we have made $X_2 = e^{(k/0.3)Y_2}$ with a point of separation $P_{X_2} = 14.303$.

In order to facilitate its use, we have constructed, on the basis of the foregoing equation, the nomogram shown in Figure 3. The small scale is for the number of drumsticks, and the large scale for the number of sessile nodules and small clubs. This nomogram permits classification directly on the basis of the number of appendices. For example, a classification corresponding to the finding of 4 drumsticks, 3 small clubs and 1 sessile nodule would be obtained as follows: 1) with the aid of a straight-edged, preferably transparent ruler, join point 4 on the small scale with point 3 on the large scale, as shown in the figure; 2) rotate the ruler about the point of intersection with the diagonal line until it touches point 1 on the large scale; 3) in the present case, the other end of the ruler would cross the small scale in the region corresponding to the female sex (*i.e.*, below the point of separation, P_{X_2}, represented by a short horizontal line) and therefore would indicate classification in that sex. Conversely, the

NOMOGRAM

How to use: The classification, for example, corresponding to a count of 4 drumsticks, 3 small clubs and 1 sessile nodule would be obtained as follows:

1. With the aid of a straightedge, preferably transparent, join the point corresponding to 4 on the small scale to number 3 on the large scale as shown in the small sketch.

2. With a pencil, mark the point where the straightedge intersects the diagonal line.

3. Rotate the straightedge on this point until it touches number 1 on the large scale.

4. In the present case the edge of the ruler crosses the small scale bellow the point of separation Px_2 (short horizontal line on small scale), thus falling in the region corresponding to individuals that are chromatin positive or female sex.

5. If the point of crossing should fall above Px_2, the classification would be chromatin negative or male sex.

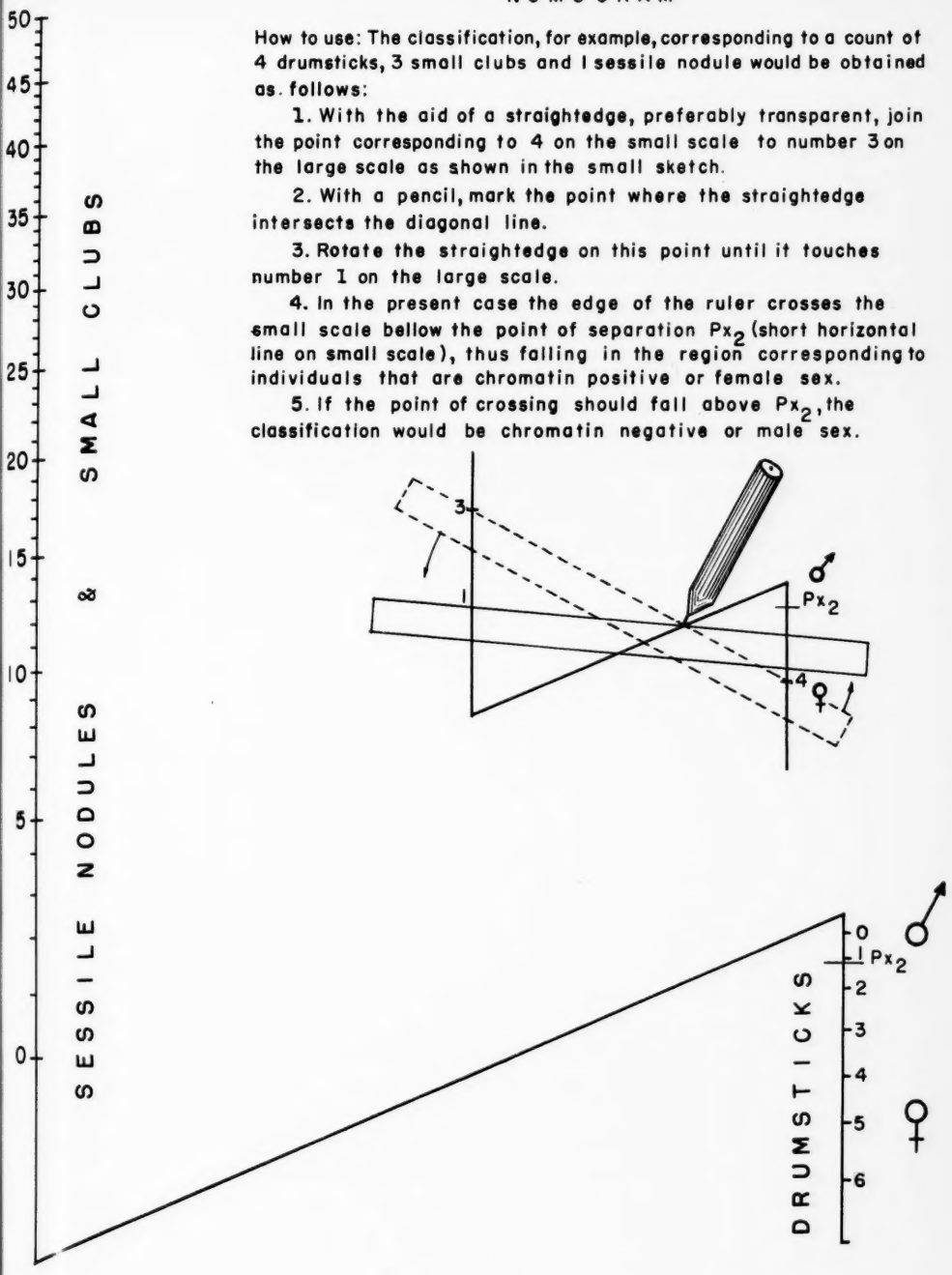

FIGURE 3

opposite classification would be indicated if the ruler crossed the line in the region corresponding to the male sex.

The scale on the nomogram shows a maximum of only 6 drumsticks, because for a subject with that number to be classified as chromatin-negative (male) the number of small clubs would have to be more than 50. Although this is theoretically possible, it would be a very rare event in practice; no such case occurred in our material. Any subject, therefore, with a count of 6 or more nuclear drumsticks should be classified as chromatin-positive (female).

Although the foregoing function seems satisfactory, there is the further possibility of eliminating y_3 (sessile nodule) and y_4 (small clubs) by making their respective coefficients equal to zero. We would then have a new function based exclusively on the number of drumsticks:

$$Y_3 = y_1$$

with averages: $\overline{Y}_{3F} = 2.065$ and $\overline{Y}_{3M} = 0.731$, point of separation $P_{Y_3} = 1.399$, and $S_{Y_3} = 0.310$. With this function, a departure of $1.399 - 0.732 = 0.667$ (or $2.066 - 1.399 = 0.667$) or 2.15 in units of standard deviation will be exceeded unilaterally, by chance, in 1.6 per cent of the cases.

Adoption of this discriminant function would serve to classify all subjects showing a value for Y_3 (or y_1) equal to or less than 1.399 (equivalent of 2 drumsticks) as chromatin-negative (male sex), and the rest (showing a count of more than 2 drumsticks) as chromatin-positive (female sex), with an error of about 1.6 per cent. Although the error of classification is slightly larger than with the previous calculations, this discriminant function is extremely simple, and depends exclusively on the number of drumsticks.

DISCUSSION AND CONCLUSIONS

The criterion currently accepted (5, 6) permits separation of a chromatin-positive group (female) with a PMN nuclear count of 6 or more drumstricks, a chromatin-negative group (male) with zero drumsticks, and a third (inconclusive) intermediate group.

In the material under study the incidence of subjects of the female sex with counts of less than 6 drumsticks per 500 PMN was 28 per cent.

Among subjects of the male sex, 9.7 per cent showed a count of 1 or more drumsticks per 500 PMN.

Thus the group of undetermined sex would include an appreciable percentage of subjects with phenotypically well-defined sex.

The existence of normal women with a low constant number of PMN nuclear drumsticks and of normal men with a count of 1 or more drumsticks must be recognized.

The currently accepted criterion would leave 18.8 per cent of subjects unclassified.

A different orientation has been followed by some other investigators (9), namely, that of counting the number of PMN until a total of 6 drumsticks has been reached, and then classifying the subjects as chromatin-positive or female regardless of the total number of PMN counted. This seems meaningless.

Adoption of the discriminant function brings most of these cases back to their correct classification, with an insignificant error.

In keeping with the criterion of Davidson and Smith (5), it is entirely correct to state that all subjects with a count of 6 or more drumsticks are chromatin-positive or female. This can be seen from the nomogram itself. In effect, a subject with a count of 6 drumsticks could only be incorrectly classified as chromatin-negative (male) if the number of small clubs were above 50—a very rare condition not once found in the material studied.

Ihm and Kosenow (24) made a discriminatory analysis of 200 normal persons, but they excluded from their material the type of case cited in the foregoing section; consequently they counted only those recognized within the currently accepted standard. In spite of the statistical accuracy achieved, they thus excluded from their study exactly those cases for which a better means of separation is needed in order to put them in their correct classification.

Discriminatory analysis of the 4 nuclear appendices as used in our study yielded highly accurate results.

Although there is the occasional possibility of confusing drumsticks with small clubs, the careful and trained observer will differentiate them better by counting them simultaneously.

The proposed nomogram is easy to use and seems to be valid, since the error is only about 0.7 per cent.

In the material studied, the majority of subjects who were classified erroneously would be so classified regardless of the discriminant function proposed, on the basis of the attributes analyzed. In most cases it was possible to find a subject of the opposite sex with the same number of nuclear drumsticks, sessile nodules and small clubs. Thus a function that would classify one correctly would classify the other incorrectly.

Our data, based on a study of 804 subjects of phenotypically well-defined sex, demonstrate great accuracy for this method of determination of sex chromatin in polymorphonuclear neutrophils.

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tedious work of tabulation and the statistical calculation. The material was collected at the Blood Bank and the Central Laboratory of the HSE, and we thank these Services for their collaboration. We also thank Dr. Halley P. Oliveira for his assistance and advice.

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EXCRETION OF FOLLICLE STIMULATING HORMONE AND INTERSTITIAL-CELL STIMULATING HORMONE IN DIFFERENT PHASES OF THE NORMAL MENSTRUAL CYCLE*

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ABSTRACT

Extracts from pools of human follicular-phase urine and pools of mid-cycle urine were compared for relative FSH and ICSH content by determining the ratio of testes weight to ventral prostate weight in hypophysectomized immature male rats. In a pilot study utilizing purified animal pituitary extracts, pituitary FSH was found to produce a significantly higher testicular-ventral prostate ratio as compared to that produced by pituitary LH. In 2 of 3 pairs of experiments there was a statistical difference in the mean regression lines produced by the extracts of mid-cycle urine as compared to those produced by follicular-phase urine. In the third experiment a trend in the same direction was noted although it could not be said that the difference was statistically significant. It was concluded that there was a qualitative difference in urinary gonadotropin excretion in the two phases of the menstrual cycle studied.

The duality of the pituitary gonadotropins hasbeen widely accepted for some time. Follicle stimulating hormone (FSH) and interstitial-cell stimulating hormone (ICSH) have been isolated separately from pituitary extracts in animals (1, 2) and more recently from human pituitary extracts (3–5). On the other hand, the existence of two separate entities, FSH and ICSH, in human urine has not been so well established.

In the past, attempts to demonstrate two separate urinary moieties have resulted in contradictory evidence. On the one hand, simultaneous assays of extracts from different sources of urine have failed to demonstrate any variation in different bio-assay systems (6–9) and this has lent strength to the belief that there may be only one molecule acting on two different end-organ systems. However, other workers (10–12) have been able to demonstrate qualitatively different biologic effects between ex-

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tracts of menopausal and male urine, although some of this variance may have come from the utilization of different methods of extraction. The best evidence for the existence of two separate urinary gonadotropins comes from a report demonstrating a separation of two distinct, qualitatively different, gonadotropic activities from urine by chromatographic methods (13).

It is the purpose of this study to explore possible qualitative differences in the gonadotropic content of urinary extracts from different phases of the menstrual cycle, *i.e.*, follicular phase and mid-cycle phase, in an attempt to throw further light on the nature of gonadotropin excretion.

MATERIAL AND METHODS

The relative FSH and ICSH contents of pituitary and urinary extracts were studied by measuring the effect of these preparations on the ratio between ventral prostate weight and testicular weight in hypophysectomized male rats. An increase in ventral prostate weight is considered fairly specific for ICSH effect (14, 15). Testicular weight responds to both FSH and ICSH (16). A relatively high ratio of testicular weight to ventral prostate weight would indicate a high content of FSH. On the other hand a low ratio of testicular weight to ventral prostate weight would indicate a relatively high content of ICSH.

Pituitary preparations.[1] FSH and LH (luteinizing hormone) obtained from the National Institutes of Health (NIH) were utilized to demonstrate the effects of relatively purified pituitary preparations on the ratio between ventral prostate and testicular weight in the test animals.

Urinary extracts. Urine specimens from normally menstruating females were pooled for comparison of the qualitative gonadotropin content of follicular-phase and mid-cycle urines. This was necessary in order to obtain sufficient amounts of extract for large enough numbers of animals to produce statistically significant results. The follicular-phase pools were prepared by combining several 24-hour urine specimens from normal volunteers, collected on the eighth and ninth days of the menstrual cycle. Approximately 8 to 10 specimens constituted one pool. The mid-cycle pools were collected from essentially normal females who were receiving insemination treatments at mid-cycle. They were requested to bring in a number of 24-hour specimens at this time. Only one such specimen was utilized in each cycle—the one associated with the thermal shift of the basal body temperature chart. This was combined with 8 or 10 others to make up the mid-cycle pool. Three pools from the follicular phase and three from the mid-cycle phase were prepared. No attempt was made to create follicular-phase and mid-cycle pools from the same subjects.

The bio-assay. Extraction and bio-assay were carried out on the urine pools according to the method described by Bradbury and Brown (17) and McArthur (18). Three dosages from each pool were administered to be certain that the differences noted were not the function of any one dose level. Whenever possible, 5 animals were utilized at each dose level. The mean ventral prostate weights and the mean combined testicular weights were determined, and the ratios of ventral prostate to testicular weight were compared.

[1] Ovine FSH and LH, generously supplied by Dr. R. T. Hill of the National Institutes of Health

TABLE 1. COMPARISON OF RATIOS OF TESTICULAR WEIGHT TO VENTRAL PROSTATE WEIGHT PRODUCED BY PITUITARY FSH AND LH

Pituitary extract	Dose (μg.)	No. of animals	Mean testes wt. (mg.)	Mean v. prost. wt. (mg.)	Ratio testes wt. / v. prost. wt.
FSH-NIH-S1	22.5	5	148	6.8	21.8
	45	5	176	6.7	25.3
	90	5	230	7.4	31.0
	180	5	270	11.0	24.6
	360	5	295	10.6	27.7
	Mean	25	224	8.1	27.7
LH-NIH-S1	7.5	5	135	9.7	13.9
	15	5	156	11.5	13.6
	30	5	168	16.5	10.2
	60	5	220	20.4	10.6
	120	5	228	27.9	8.2
	Mean	25	181	17.2	10.5

RESULTS

Figure 1 illustrates the effect of increasing doses of NIH animal pituitary LH and FSH on the ventral prostate weight and testicular weight of the test animals. Pituitary LH caused a relatively large increase in the weight of the ventral prostate as compared to the weight of the testes, and pituitary FSH caused the reverse effect, in that there was a relatively larger increase in testicular weight as compared to ventral prostate weight. Indeed, pituitary FSH apparently caused little, if any, increase in the weight of the ventral prostate, and whatever increase did occur may well have been due to contamination with LH.

This difference in effect can be mathematically expressed as the ratio of testicular weight to ventral prostate weight. Table 1 lists the mean ventral prostate and testicular weights together with the ratios of testicular weights to ventral prostate weights produced by the different doses of animal pituitary LH and FSH utilized for the preceding dose-response curves. The over-all mean testicular-ventral prostate ratio produced by pituitary LH was 10.5, whereas that produced by pituitary FSH was 27.7.

For purposes of comparing these ratios statistically, the individual organ weights were converted to logarithms and the regression of the logarithm of ventral prostate weight on the logarithm of testicular weight was plotted for both LH and FSH as shown in Figure 2. Only the 3 higher dose levels were utilized in order to be certain that an adequate stimulation of both testes and ventral prostate weight had been obtained. The 95 per cent confidence belts, calculated for both regression lines, were also charted. The fact that the confidence bands do not overlap would in-

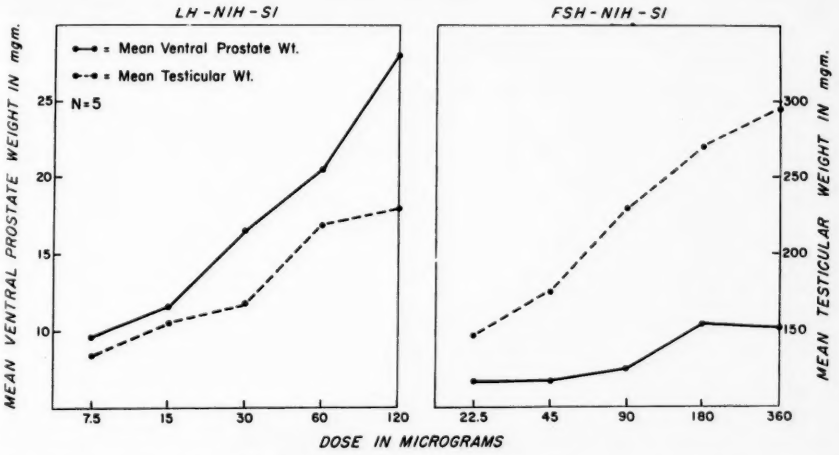

FIG. 1. Effect of pituitary LH and FSH (NIH) on ventral prostate weight and testicular weight in hypophysectomized immature male rats.

dicate a statistically significant difference between the mean responses at the dose levels studied. It is apparent that administration of pituitary LH resulted in a relatively larger unit of ventral prostate weight per unit of testicular weight as compared to pituitary FSH.

The 3 follicular-phase pools and the 3 mid-cycle pools were similarly compared. Groups of animals were given three-hour, six-hour and nine-hour equivalents of extract, respectively. These doses were chosen because they had been shown by McArthur (18) to produce a significant increase in the weight of both the testes and ventral prostate. An attempt was made to utilize 5 animals at each dose level, but due to toxicity of the extracts not all animals survived. In Table 2 are listed the respective mean testicular weights and ventral prostate weights (based on measurements of from 2 to 5 rats) which were considered to be significantly different from control weights ($P = 0.05$). In addition, there was some variation in the potency of the pools. For purposes of comparison, pairs of follicular-phase and mid-cycle pools were selected for which the mean testicular weights

TABLE 2. UPPER 95% CONFIDENCE LIMIT FOR 27 CONTROL ANIMALS
(mean ventral prostate wt. = 6.5 mg. ± 1.52; mean testes wt. = 138 mg. ± 5.71)

No.	V.P. wt. (mg.)	Testes wt. (mg.)
2	10.3	152
3	7.8	143
4	7.5	142
5	7.3	141

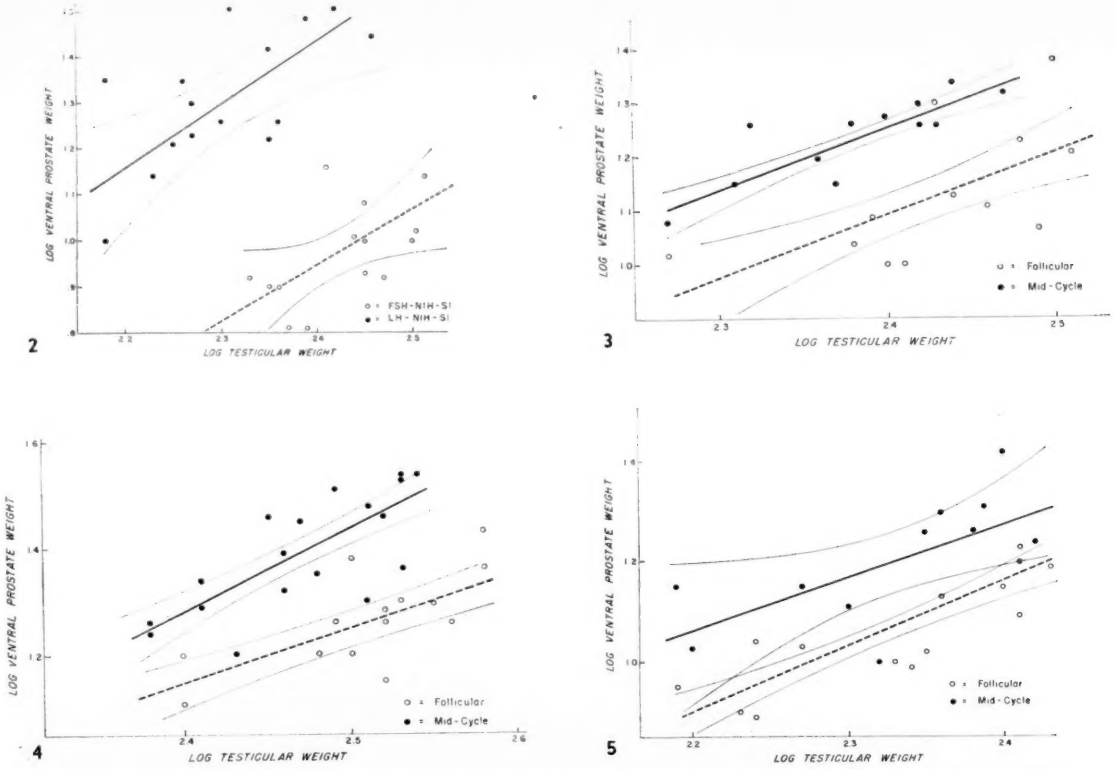

FIG. 2. Relationship between testicular and ventral prostate weights produced by pituitary FSH and LH. The solid points represent individual observations produced by LH stimulation, and the solid line represents the mean regression line for these points. The circles represent the individual observations produced by FSH, and the dotted line represents the mean regression line for these points.

FIG. 3. *Series 1.* Relationship between testicular and ventral prostate weights produced by extracts from pooled human follicular-phase and mid-cycle urine.

FIG. 4. *Series 2.* Corresponding relationship.

FIG. 5. *Series 3.* Corresponding relationship.

TABLE 3. COMPARISON OF RATIOS OF TESTICULAR WEIGHT TO VENTRAL PROSTATE WEIGHT PRODUCED BY POOLS OF HUMAN FOLLICULAR-PHASE AND MID-CYCLE URINE—SERIES 1

Pool	Dose in hr.-equivs. of extract	No. of animals	Mean testes wt. (mg.)	Mean v. prost. wt. (mg.)	Ratio testes wt. / v. prost. wt.
Follicular-phase	3	2	213	10.7	21.4
	6	5	265	12.8	20.7
	9	5	292	16.7	17.9
	Mean	12	266	14.1	18.9
Mid-cycle	3	5	220	15.2	14.5
	6	5	248	18.2	13.6
	9	3	278	21.0	13.1
	Mean	13	244	17.7	13.7

were most in agreement. This would indicate a rough agreement in total gonadotropin potency.

Table 3 shows the mean testicular and ventral prostate weights together with the testes-ventral prostate ratio for the first pair (Series 1) of follicular-phase and mid-cycle pools. Figure 3 demonstrates the mean regression line of the logarithms of the individual weights of these 2 pools together with 95 per cent confidence bands calculated for these mean regression lines. Within the range of the observations there was only minor overlapping of the bands at the extreme ends, and this would indicate a statistically significant difference in the mean regression lines.

Table 4 shows the weights and ratios for the second pair of pools (Series 2). The over-all mean testes-ventral prostate ratio for the follicular-phase pool was 18.5, whereas the over-all mean ratio for the mid-cycle pool was 11.6. The logarithms of the individual observations, the mean regression

TABLE 4. COMPARISON OF RATIOS OF TESTICULAR WEIGHT TO VENTRAL PROSTATE WEIGHT PRODUCED BY POOLS OF HUMAN FOLLICULAR-PHASE AND MID-CYCLE URINE—SERIES 2

Pool	Dose in hr.-equivs. of extract	No. of animals	Mean testes wt. (mg.)	Mean v. prost. wt. (mg.)	Ratio testes wt. / v. prost. wt.
Follicular-phase	3	5	293	15.0	19.5
	6	5	327	17.8	18.4
	9	5	355	2.01	17.7
	Mean	15	325	17.6	18.5
Mid-cycle	3	6	241	19.8	12.1
	6	6	294	23.8	12.4
	9	6	325	32.2	10.4
	Mean	18	293	25.3	11.6

lines, and 95 per cent confidence bands are plotted in Figure 4. Once again the confidence belts did not overlap and there was statistical evidence that the extract from the mid-cycle urine produced testes-ventral prostate ratios different from those produced by the extract from the follicular-phase urine.

The mean weights for each dose group and testes-ventral prostate ratios for the third pair of pools (Series 3) are listed in Table 5. The over-all mean testes-ventral prostate ratio for the follicular-phase pool was 18.0, and the over-all mean ratio for the mid-cycle pool was 12.9. This difference was of somewhat the same order as in the previous pairs. However, due to the

TABLE 5. COMPARISON OF RATIOS OF TESTICULAR WEIGHT TO VENTRAL PROSTATE WEIGHT PRODUCED BY POOLS OF HUMAN FOLLICULAR-PHASE AND MID-CYCLE URINE—SERIES 3

Pool	Dose in hr.-equivs. of extract	No. of animals	Mean testes wt. (mg.)	Mean v. prost. wt. (mg.)	Ratio testes wt. / v. prost. wt.
Follicular-phase	3	5	172	9.3	18.5
	6	4	228	11.7	19.5
	9	5	254	14.9	16.9
	Mean	14	216	12.0	18.0
Mid-cycle	3	5	182	12.4	14.6
	6	4	240	19.0	12.6
	9	2	246	22.2	11.8
	Mean	11	197	15.2	12.9

wider scattering of the observations there was some overlapping of the 95 per cent confidence bands, as demonstrated in Figure 5. This indicated that the results were not statistically significant and that only a trend in the direction of a difference could be demonstrated.

DISCUSSION

The fact that statistically significant qualitative differences in gonadotropin activity could be demonstrated in at least 2 of 3 mid-cycle extracts of urine as compared to follicular-phase extracts would lend support to the dual concept of urinary gonadotropins. It is fair to assume that if a more accurate method of selecting the exact mid-cycle day were available these differences might have been more marked, and statistically significant in all 3 instances.

The failure of previous workers (8) to demonstrate qualitatively different biologic effects between follicular-phase and mid-cycle urine has undoubtedly been due to the fact that collections for consecutive days were utilized for the mid-cycle pool, instead of pooling individual mid-cycle

days as in the present study. The effect of the consecutive days apparently serves to dampen the qualitative differences and render them nondetectable.

An assay method involving the use of two end-organs within the same animal reduces the amount of total extract required for the studies and eliminates the variables produced by different dilutions, injections and variations in animal absorption. Thus, each individual pool of urine was handled in exactly the same manner as the others; the only difference was the source. Since a qualitatively different result was produced, one can conclude that qualitative differences existed between the pairs of pools, *i.e.*, between mid-cycle and follicular-phase urine. If a qualitative difference exists, in all likelihood more than one factor is present.

The use of ratios to compare qualitative differences in hormone effect has the added advantage of making it unnecessary to use a standard. A standard is needed to calculate levels of hormone excretion for purposes of quantitative comparison with other assays, and to take into account the day-to-day and week-to-week variations in animal responsiveness. A quantitative determination of hormone excretion was not needed in this study. Furthermore, it is unlikely that there would be an individual change in organ responsiveness (testes without ventral prostate) purely on the basis of total change in animal responsiveness. Therefore, a comparison of mean ratios for testes-ventral prostate weights in groups of animals is probably valid without the use of a standard, even when the groups of animals were tested at different time intervals.

The fact that the effect of mid-cycle urine more closely resembled that of pituitary LH, and the effect of follicular-phase urine more closely resembled that of pituitary FSH would confirm the long-held concept that the mid-cycle peak of gonadotropin excretion is indeed accompanied by a relative increase in LH or ICSH over FSH.

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OXYTOCIC ACTIVITY OF BLOOD IN PARTURIENT AND LACTATING WOMEN

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ABSTRACT

The Bristol procedure was used for the extraction of oxytocin from the blood taken from healthy pregnant women during the last trimester of pregnancy and from women during labor. Both oxytocin and oxytocic substance (O.S.) were found in the plasma extracts. The levels of oxytocin in the blood were the same during labor as during late pregnancy, but more O.S. was present in early labor than during pregnancy. The concentration of O.S. was higher in early labor than in late labor. There were no differences in the oxytocic activity (O.A.) of blood samples drawn (a) during and between uterine contractions, and (b) from primiparous and multiparous women. Precautions were taken to exclude the possibility that potassium, 5-hydroxytryptamine, acetylcholine and plasma kinin in the acetone extracts might be acting as interfering substances in the assays. The extracts were assayed on the isolated sensitized rat uterus, by the use of both the superfusion and classic methods. The results obtained suggest that O.S., rather than oxytocin, may be involved in the mechanism of onset of parturition. Samples of blood taken from healthy lactating women who had babies 1–5 months old were assayed for oxytocin as described. Both oxytocin and oxytocic substance contributed to the oxytocic activity of these extracts. The levels of O.A., oxytocin and O.S. were determined in the extracts of blood samples taken immediately before and during suckling from: 1) women who were wholly, and those who were partly, breast-feeding their infants; 2) primiparous and multiparous women, and 3) women who has been lactating for less than six weeks, and those lactating for more than six weeks. There was no difference between the blood oxytocin levels before and during suckling, but the levels of O.A., oxytocin and O.S. were higher in nursing women than in those who were not nursing. There were no statistical differences in the levels of O.A., oxytocin and O.S. in blood taken from women who were wholly breast-feeding and those who were complementing their babies' diets, nor were there any differences in the levels of these 3 substances between primiparous and multiparous women.

THE mechanism for the onset of labor has not been elucidated but it has been postulated that during labor more oxytocin is released into the circulation and that, in response to this stimulation, uterine activity

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increases (1). It seemed pertinent to investigate the possible causal role of oxytocin in labor. We have previously reported finding oxytocin and oxytocic substance (O.S.) in extracts of blood from women in both non-pregnant and pregnant states (2, 3). In the earlier study (2) oxytocin was extracted from blood by the acid-alcohol method described by Bisset and Walker (4); however, since this method entails acidification, a process which is known to liberate oxytocic polypeptides from the plasma proteins (5, 6), it was abandoned in favor of the Bristol procedure (7) which has been employed in recent studies (3) and in this investigation.

We determined the oxytocic activity (O.A.) of blood taken from women during pregnancy and during various stages of labor. The results obtained do not support the postulate that oxytocin is involved in the onset of parturition.

As a sequel to these studies, we investigated the oxytocic activity of the blood of lactating women. It is generally accepted that suckling stimulates the ejection of milk, by means of a neurohumoral reflex; impulses pass from receptors in the mammary gland to hypothalamic nuclei, to initiate the release of oxytocin which facilitates the expression of milk (8–10).

Bisset and Walker's method for the extraction of oxytocin from blood was used in a study in which we determined the oxytocin levels in blood taken from cows, goats and the human female before, during and after manipulation or suckling of the mammary gland (11–13). Our results for cows (11) showed that the blood level of oxytocin immediately before milking was virtually the same as that during milking, whereas three hours later it was much lower. This suggested that a conditioned release of oxytocin had occurred in response to environmental stimuli associated with the milking routine. In goats, the level of oxytocin in the blood was found to rise during milking. In both species there was practically no oxytocin in the blood three hours after milking when the animals were away from the bails.

Studies on lactating women (12, 13) indicated that the oxytocin content of the mother's blood did not appear to alter whether the blood was taken immediately prior to, during, or midway between four-hourly feedings.

In the present investigation the Bristol procedure (7) for extraction of oxytocin from blood was used to determine the oxytocic activity (O.A.) of blood taken from women immediately before and during suckling. The results obtained confirm our previous observations that the oxytocin level in the blood is as high immediately before suckling as during suckling.

METHODS

Preparation of the extracts

The Bristol procedure for the extraction of oxytocin from blood (7) is briefly described as follows: Samples of venous blood were collected from the antecubital veins

of the various subjects and prevented from clotting by adding 100 I.U. of heparin in 1.0 ml. of isotonic sodium chloride to every 10-ml. sample of blood. The blood was drawn from the vein through a siliconed needle into an ice-cold polythene syringe and then put into ice-cold polythene tubes, centrifuged at 0°C., and the plasma removed. Ten volumes of acetone were added to the plasma to precipitate the proteins. At this stage the supernatant was stored overnight in the deep-freeze and extraction was continued next morning. The supernatant was reduced by vacuum distillation to remove the acetone. The residue was washed twice with three volumes of ether, which was removed by suction. Residual ether was blown off at 37°C. with nitrogen and the extracts were assayed at pH 7.4. Polythene apparatus was used, to prevent the possible formation of plasma kinin which may act as an interfering oxytocic principle.

The blood samples were collected from women attending the Woolloongabba Centre of the Maternal and Child Welfare Department, Brisbane, and the Women's Hospital, Herston, Brisbane.

Method of assay

The extracts were assayed in duplicate for O.A. against Pitocin (Parke, Davis & Co.) and at times against synthetic oxytocin, Syntocinon (Sandoz Ltd.), by the use of the superfused rat-uterus technique as described by Gaddum (14). In every instance, residual oxytocic activity could be demonstrated after treatment of the extracts with both 0.01 *M* and 0.002 *M* sodium thioglycollate (7, 15, 16). This activity was attributed to an unidentified factor which we have referred to as oxytocic substance (O.S.). The possibility that O.S. was acetylcholine or 5-hydroxytryptamine could be excluded by adding atropine to the perfusing fluid and administering small doses of Dibenamine hydrochloride at the beginning of the assay. It was evident from flame photometry estimation that the potassium concentration in test doses was sub-threshold and therefore could not account for the residual activity.

In most of the studies involving women in labor, the extracts were assayed in parallel by use of both the superfused uterus and the uterus in the classic organ bath as described by Holton (17) and Gaddum, Peart and Vogt (18).

RESULTS

Women in labor

The oxytocic activity (O.A.) of extracts of blood from 35 women during labor is shown in Table 1, together with data on the parity and degree of cervical dilatation in the women investigated. It will be seen that the O.A. was due to the presence of both oxytocin and O.S. in the extracts, and also that the mean ratio of oxytocin to O.S. was approximately 1:2.7.

Table 2 shows the O.A. of acetone extracts of blood taken during the third trimester of pregnancy; here the mean ratio of oxytocin to O.S. was 1:1.7. It may be seen that the mean O.A. of the extracts of blood taken during labor did not differ significantly from that in the third trimester of pregnancy. Neither was there any significant difference in the two mean values for oxytocin; however there was significantly more O.S. in the extracts of blood obtained in early labor than during late pregnancy ($P = 0.013$). Incidentally, there is no inconsistency in having differences in O.S. that are significant while the differences in O.A and oxytocin are not

TABLE 1. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF BLOOD FROM WOMEN IN LABOR (mU/ML. BLOOD)

No.	Parity*	Dilatation of cervix†	Oxytocic activity	Oxytocin	Oxytocic substance
1	P	$<\frac{1}{2}$	9.92	5.95	3.97
2	M	$<\frac{1}{2}$	2.04		
3	M	$>\frac{1}{2}$	0.26		
4	M	$>\frac{1}{2}$	0.57		
5	M	$>\frac{1}{2}$	13.84		
6	M	$>\frac{1}{2}$	16.62		
7	M	$>\frac{1}{2}$	4.48		
8	M	$>\frac{1}{2}$	1.67	0.67	1.00
9	M	$>\frac{1}{2}$	1.51	0.36	1.15
10	P	$<\frac{1}{2}$	0.34		
11	P	$<\frac{1}{2}$	0.40		
12	M	$<\frac{1}{2}$	0.95	0	1.08
13	M	$<\frac{1}{2}$	1.43		
14	P	$>\frac{1}{2}$	0.63		
15	P	$>\frac{1}{2}$	1.71		
16	M	$<\frac{1}{2}$	4.66		
17	P	$<\frac{1}{2}$	2.35		
18	M	$>\frac{1}{2}$	2.85	2.54	0.31
19	M	$>\frac{1}{2}$	3.47	1.51	1.96
20	P	$<\frac{1}{2}$	5.17	1.17	4.00
21	P	$<\frac{1}{2}$	6.30	0.95	5.35
22	M	$>\frac{1}{2}$	1.83		
23	M	$>\frac{1}{2}$	2.39	0.43	1.96
24	P	$<\frac{1}{2}$	2.08		
25	P	$<\frac{1}{2}$	6.14	0.97	5.17
26	P	$<\frac{1}{2}$	13.59	0	14.18
27	P	$<\frac{1}{2}$	9.34	3.74	5.60
28	M	$<\frac{1}{2}$	8.33	1.13	7.20
29	P	$>\frac{1}{2}$	2.70	0.17	2.53
30	P	$>\frac{1}{2}$	1.90	0.44	1.46
31	P	$<\frac{1}{2}$	6.17	2.17	4.00
32	P	$<\frac{1}{2}$	5.25		
33	P	$<\frac{1}{2}$	5.46	2.16	3.30
34	P	$<\frac{1}{2}$	3.08	0.63	2.45
35	M	$>\frac{1}{2}$	4.78		
Mean			4.41	1.39	3.70
S.D.			4.02	1.47	3.14
S.E. of the mean			0.68	0.35	0.74
Fiducial limits (P = 0.05)			3.30–5.79	0.65–2.13	2.14–5.26

* Primiparous; M = multiparous.
† $<\frac{1}{2}$ = less than 5 cm. dilatation; $>\frac{1}{2}$ = more than 5 cm. dilatation.

significant. The O.S values were possibly subject to less variation owing to differences in the number of observations made. A glance at Tables 1 and 2 will disclose the wide standard deviations obtained for O.A on both groups of women.

We have previously shown (2, 3) that the O.A. of blood is higher during pregnancy than in the nonpregnant state. This increase in activity occurs early in pregnancy and continues until the second trimester; thereafter the level remains high until the end of pregnancy. It follows that there is

TABLE 2. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF BLOOD FROM PREGNANT WOMEN (mU/ML. BLOOD)

No.	Oxytocic activity	Oxytocin	Oxytocic substance
1	11.2	7.0	4.2
2	5.33	1.33	4.0
3	0.14	0.02	0.12
4	1.03	0.29	0.74
5	9.41	3.50	5.91
6	1.75	0.44	1.31
7	0.34	0.13	0.22
8	3.03	0.93	2.10
9	0.41	0.09	0.33
10	0.64	0.36	0.28
11	1.87	0.41	1.62
12	3.17	1.14	2.28
13	0.12	0.09	
14	8.0		
15	1.50		
16	0.11		
17	0.04		
18	0.53		
Mean	2.70	1.10	1.92
S.D.	3.37	1.74	1.98
S.E. of mean	0.79	0.44	0.54
Fiducial limits (P=0.05)	1.03–4.37	0.17–2.03	0.73–3.11

more O.A. in the blood throughout labor than during either the non-pregnant state or the first half of pregnancy.

Table 3 shows that there is no significant difference in the O.A. of blood taken during and between uterine contractions in the same women.

TABLE 3. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF BLOOD FROM WOMEN IN LABOR (mU/ML. BLOOD)

No.	Oxytocic activity	
	Before contraction	During contraction
1	0.26	0.57
2	13.84	16.62
3	4.48	4.78
4	1.67	1.51
5	0.40	0.34
6	1.43	0.95
7	0.63	1.71
8	8.33	4.66
9	2.85	3.47
10	6.30	5.17
11	1.83	2.39
12	6.14	2.08
13	13.59	9.34
14	1.90	2.70
15	6.17	5.25
16	5.46	3.08
Mean	4.71	4.04
S.D.	4.16	3.93
S.E. of mean	1.04	0.98
Fiducial limits (P=0.05)	2.49–6.93	1.95–6.13

We have arbitrarily classified women to be in early or late labor according to whether the cervix was dilated less than or more than 5 cm. A comparison of the O.A. of blood samples taken during early and late labor, showed that there was no significant difference between the two. The oxytocin values did not differ significantly in these two groups, but there was significantly more O.S. in early labor than in late labor ($P = 0.004$).

There also was no significant difference in the O.A., oxytocin and O.S.

TABLE 4. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF BLOOD FROM WOMEN DURING LACTATION (mU/ML. BLOOD)

No.	Condition*	Oxytocic activity		Oxytocin		Oxytocic substance	
		5 minutes before	during	5 minutes before	during	5 minutes before	during
1	P, FB, 18	0.38	0.23	0.10	0.05	0.28	0.18
2	P, PB, 4	0.40	0.27	0.06	0.13	0.34	0.14
3	P, PB, 10	6.77	1.88	1.77	0	5.00	1.92
4	P, PB, 5	0.10	2.31				
5	P, PB, 4	0.64		0.35		0.29	
6	M, PB, 16	0.20	0.14	0.10	0.06	0.10	0.08
7	P, PB, 12	0.59	2.20	0.12	1.67	0.47	0.53
8	M, FB, 6	0.13	0.12	0.08	0.05	0.05	0.07
9	M, FB, 10	0.45	12.5	0.09	7.70	0.36	4.80
10	P, FB, 18	0.37	0.64	0	0	0.37	0.64
11	P, FB, 9	0.30	0.16	0.08	0.03	0.22	0.13
12	M, FB, 7	6.00	0.23	4.40	0.10	1.60	0.13
13	P, FB, 4	0.06	0.55		0.22		0.33
14	P, PB, 15	0.05	0.07				
15	P, FB, 17	0.24	0.10	0.12	0.02	0.12	0.08
16	P, FB, 5	0.32	0.21	0.20	0.02	0.12	0.19
17	P, FB, 6	2.70	1.31		0.35		0.96
18	M, PB, 19	0.24	0.20	0.12	0.004	0.12	0.20
19	M, PB, 21	0.06	0.07				
20	M, FB, 3	0.05	0.14				

* P = primiparous; M = multiparous; FB = fully breast fed; PB = partly breast fed; the number indicates weeks of lactation.

content of extracts of blood, when comparing primiparous and multiparous women.

Lactating women

Table 4 shows the O.A. of acetone extracts of blood from women during lactation in milliunits (mU) per ml. of blood This series consisted of primiparous and multiparous women, some of whom were wholly breast-feeding their infants. It is evident from the table that the O.A. of the extracts was due to the presence of both oxytocin and O.S.

Reference to Table 5 reveals that the mean values for O.A., oxytocin and O.S. in the extracts of blood taken from women immediately before a regular breast-feeding time were 1.02, 0.54 and 0.67 mU per ml.; the corresponding values for the same women during expression of milk were

TABLE 5. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF BLOOD FROM WOMEN DURING LACTATION (mU/ML. BLOOD): STATISTICAL RESULTS

		Mean	S.D. of mean	S.E. of mean	Fiducial limits (P=0.05)
5 Minutes before suckling	Oxytocic activity	1.02	1.93	0.44	0.10-1.94
	Oxytocin	0.54	1.15	0.31	0 -1.21
	Oxytocic substance	0.67	1.26	0.34	0 -1.40
During suckling	Oxytocic activity	1.23	2.75	0.63	0 -2.55
	Oxytocin	0.72	1.91	0.49	0 -1.78
	Oxytocic substance	0.69	1.20	0.31	0.02-1.36
Fully breast fed	Oxytocic activity	1.24	2.77	0.59	0.01-2.47
	Oxytocin	0.76	1.95	0.46	0 -1.73
	Oxytocic substance	0.59	1.09	0.26	0.04-1.14
Partly breast fed	Oxytocic activity	0.95	1.63	0.40	0.10-1.80
	Oxytocin	0.44	0.62	0.19	0.02-0.86
	Oxytocic substance	0.84	1.40	0.42	0 -1.78
Primiparous	Oxytocic activity	0.91	1.24	0.25	0.40-1.42
	Oxytocin	0.29	0.50	0.11	0.06-0.52
	Oxytocic substance	0.65	1.10	0.25	0.13-1.17
Multiparous	Oxytocic activity	1.47	3.41	0.91	0 -3.44
	Oxytocin	1.27	2.50	0.79	0 -3.06
	Oxytocic substance	0.75	1.42	0.45	0 -1.77
Early lactation	Oxytocic activity	0.62	0.81	0.21	0.17-1.07
	Oxytocin	0.16	0.10	0.03	0.09-0.23
	Oxytocic substance	0.28	0.26	0.09	0.07-0.49
Late lactation	Oxytocic activity	1.42	2.88	0.59	0.20-2.64
	Oxytocin	0.85	1.88	0.42	0 -1.73
	Oxytocic substance	0.84	1.41	0.32	0.17-1.51
Lactation (over-all results)	Oxytocic activity	1.11	2.35	0.38	0.34-1.88
	Oxytocin	0.64	1.59	0.29	0.05-1.23
	Oxytocic substance	0.67	1.21	0.22	0.22-1.12

1.23, 0.72 and 0.69 mU per ml. of blood. There was no statistical difference between the total activity, the oxytocin and O.S. values of the extracts of blood taken before and during suckling.

In the women who were wholly breast-feeding their infants the mean values for O.A., oxytocin and O.S. content of blood were 1.24, 0.76 and 0.59 respectively, and these did not differ significantly from the mean values (0.95, 0.44 and 0.84 respectively) in the women who were complementing the infant diet. These figures were derived from the mean of 2 readings for each woman, obtained before and during suckling.

A comparison of primiparous with multiparous lactating women without regard to whole or part breast-feeding revealed no significant differences in the values for O.A., oxytocin and O.S. In the primiparous lactating women, the mean values for O.A., oxytocin and O.S. were 0.91, 0.29 and 0.65

mU per ml. of blood, whereas in the multiparous series the corresponding mean values were 1.47, 1.27 and 0.75.

We arbitrarily divided our series of lactating women into "early" and "late"; the former term signified that the lactation had progressed for less than six weeks, and the latter, for more than six weeks. In the early series the mean values for O.A., oxytocin and O.S. were 0.62, 0.16 and 0.28 mU per ml. of blood, and in the late series the corresponding mean values were 1.42, 0.85 and 0.84 mU.

The over-all results in all the women investigated during lactation for O.A., oxytocin and O.S. were 1.11, 0.64 and 0.67 mU per ml. of blood; comparable values for nonpregnant women were 0.42, 0.12 and 0.38 respectively (3). Thus significantly more oxytocin ($P=0.001$) and O.S. ($P=0.02$) are found in the blood of lactating women than in the blood of nonpregnant women.

DISCUSSION

Women in labor

The O.A. of the acetone extracts was higher in blood drawn from women during labor than in nonpregnant women, confirming an observation previously reported by Hawker and Robertson (2). The O.A. during labor was also higher than during the early stages of pregnancy (3). It is noteworthy that there was no significant difference in the O.A. of the blood extracts between women in labor and women in late pregnancy, nor was there any difference in the oxytocin levels between these groups. However, there was significantly more O.S. in early labor than during the later months of pregnancy. Moreover, the levels of oxytocic activity were the same during uterine contractions and between contractions.

Caldeyro-Barcia and Sereno (1) have shown that during pregnancy the uterus becomes increasingly sensitive to oxytocin until about the thirty-fourth week of pregnancy. From then on, until the onset of labor and during labor, there is no change in the sensitivity of the uterus to oxytocin. These authors postulated that during labor more oxytocin is secreted into the blood from the hypothalamico-posterior pituitary complex. They suggested that early in labor the hypothalamico-posterior pituitary region liberates about 2 milliunits of oxytocin per minute, and that as labor progresses up to the end of the second stage, the output of oxytocin increases to about 8–16 milliunits per minute. They based these assumptions on studies of the pressure changes of the amniotic fluid in the pregnant human uterus in response to intravenous infusion of oxytocin. Their findings are in accord with the reflex response described by Ferguson (19), who observed that dilatation of the uterus, but more especially of the cervix, acts as a secretory stimulus on the neurohypophysis. Within the limits of

ensitivity of our method of assay, our results do not support the observations of Caldeyro-Barcia and Sereno, nor can they be explained in terms of the Ferguson reflex, since we found the same oxytocin level in the blood in early labor as in late labor. However, we found significantly more O.S. in the blood during early labor than during late labor. This would appear to indicate that O.S. may be involved in the mechanism of the onset of labor, either as a parent or precursor substance, a derivative, or a breakdown product of oxytocin itself.

O.S. does not seem to be an artefact of the extraction method used in this investigation, because (a) precipitation of whole blood was avoided, thus eliminating the release from the blood cells of known and unknown stimulants of smooth muscle which cannot be antagonized efficiently; (b) the method does not entail acidification, which is known to split off oxytocic polypeptides (5, 6); and (c) there is no liberation of plasma kinin (or pain-producing substance), as assured by the use of polythene and siliconed apparatus throughout. It is unlikely that the treatment of extracts with sodium thioglycollate creates an artefact, since (a) the pH is maintained at 7.4; (b) the specific effect of thioglycollate is simply to disrupt disulphide bonds (15); and (c) sodium thioglycollate has no stimulating effect on the rat uterus except in amounts larger than those employed in these studies. The oxytocic substance that accounts for the residual activity is unlikely to be oxytocin, since sodium thioglycollate destroys 99.5 per cent of the oxytocic activity of Pitocin (Parke, Davis & Co.) after two to five minutes of treatment (15).

Lactating women

It is generally accepted that during suckling oxytocin facilitates the expression of milk. In the cow and goat, oxytocin is released before milking occurs, apparently in response to a conditioned reflex which develops initially as a result of auditory, visual and olfactory perceptions associated with milking (11).

In the course of previous studies (12), it was shown that, in women with babies 4–7 days old, the O.A. of the mother's blood generally increased during suckling and that this was due mainly to O.S. and not to oxytocin.

Later, it was found that, in women whose babies were 12 to 28 weeks old, there was no change in the O.A. (consisting chiefly of O.S.) of blood collected immediately prior to suckling, during suckling or midway between four-hourly feedings (13).

In the present investigation (the babies were fed at a routine time), the results indicate that in the human female, whether wholly or partly breast-feeding her infant and regardless of parity, there is no observable difference in the blood levels of oxytocin before and during suckling. Since there is

also no statistical difference between the oxytocin levels in women who have been lactating for more than six weeks and those for less than six weeks, it would appear that the oxytocin level rises early in lactation and remains high. However this assumption could be verified only by obtaining these high levels of oxytocin at all times in women in whom lactation is established.

It was of interest that no significant differences existed between the blood oxytocin levels in women who were wholly breast-feeding, and those who were partly breast-feeding their infants, nor between the levels in primiparous and multiparous lactating women.

In a recent investigation the mean values for O.A., oxytocin and O.S. in blood samples from 13 nonpregnant women were 0.42, 0.12 and 0.38 mU per ml. respectively (3). The blood of the lactating women in the present investigation who were accustomed to feeding their babies on a time schedule contained significantly more oxytocin ($P = 0.001$) and O.S. ($P = 0.02$) than was found in our series of nonpregnant women.

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ENDEMIC GOITER IN THE UELE REGION. II. SYNTHESIS AND SECRETION OF THYROID HORMONES*

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ABSTRACT

A study was made of the labeled iodinated amino acids and the specific activities of organic iodine in the thyroid and blood of 14 goitrous euthyroid patients of the Uele endemic area (Africa). Partial thyroidectomy was performed three to ten days after administration of I^{131}. The total iodine content of the thyroid was in the normal range, but the concentration of iodine per gram was reduced to about 25 per cent. Although the thyroidal uptake of I^{131} was increased in all the patients, the level of plasma PBI^{131} was low in some cases (0.05 per cent of the dose per liter at twenty-four hours), and much higher in others (0.44 per cent). In the 8 "low PBI^{131}" patients the isotopic equilibrium between the thyroidal and extrathyroidal organic iodine pools had not been reached seven days after administration of I^{131}, whereas in the 6 "high PBI^{131}" patients the specific activity of the organic iodine was two to seven times higher in the blood than in the thyroid gland. In the first group, there was a slowing down of the hormonal secretion rate. In the second group, the high PBI^{131} levels suggest the existence of an iodine pool with a fast turnover, functionally different from the rest of the thyroidal iodine stores. Chromatographic analysis of thyroid tissue after hydrolysis showed an increase of the MIT^{131}/DIT^{131} ratio and a decrease in the labeled iodothyronine fraction. This distribution was probably the result of a diminution in the rate of transfer of I^{131} into the more heavily iodinated compounds. From this study it appears that besides an increased thyroidal iodine uptake, severe iodine deficiency induces several abnormalities in the synthesis and excretion of the thyroid hormone.

RECENT experimental work (1, 2) has shown that a prolonged iodine-deficient diet induces modifications in the iodinated amino-acid content of the thyroid gland. Similar information is not available at the present

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time regarding thyroid hormonogenesis in endemic iodine-deficient goiter. However, the results of previous studies (3–6) suggest that in this condition, alterations of the secretion pattern may occur.

The present investigation deals with the synthesis and secretion of thyroid hormone in goitrous patients of the Uele region (Central Africa). Clinical and biologic studies (7, 8) of this form of endemic goiter have emphasized the severity of the iodine deficiency in this area as assessed by the low concentration of iodine in the drinking water, the low urinary excretion of iodine, and the low level of protein-bound iodine in the blood.

Goitrous Uele patients who underwent partial thyroidectomy were studied before and after operation by radioiodine techniques, including the chromatographic analysis of the labeled iodinated amino acids and the determination of the specific activity of the organic iodine in the blood and in the removed thyroid gland.

MATERIAL AND METHODS

Patients

Three male and 11 female goitrous subjects, aged from 14 to 42, were studied in the Poko district of the Uele goitrous endemic area. All were considered euthyroid by the usual clinical criteria and had not received iodine therapy during the previous three years.

The selected patients were those scheduled to undergo surgical treatment for diffuse or nodular nontoxic goiter. A dose of 500–1000 microcuries of carrier-free iodine^{131} (Centre d'Energie Nucléaire, Mol) was administered by mouth three to ten days before partial thyroidectomy. The thyroidal uptake of I^{131} was determined by external scintillation counting over the gland. Blood samples were withdrawn for determination of stable and radioactive protein-bound iodine at twenty-four and forty-eight hours after administration of I^{131} and immediately before the operation.

Five Belgian patients aged from 38 to 56 years, having no thyroid disorders and about to undergo an operation on the neck for laryngeal carcinoma, were used as controls. In 3 of the 5, biopsy of the thyroid was performed forty-eight hours after the the administration of 100 microcuries of I^{131}.

Analysis of thyroidal iodine

After removal, the thyroid glands were immediately frozen and sent by air to Belgium; during the trip the temperature never exceeded −5°C. The glands were then homogenized (Potter-Elvehjem) and aliquots were used for determination of radioactive and stable iodine. Radioiodine was measured by means of a well-type scintillation counter and compared with the radioactivity of a suitable dilution of the administered dose of I^{131}. Percentile uptakes of radioiodine per gram of tissue were calculated. Chemical assays of protein-bound iodine in thyroid tissue were performed either after the technique of Grossman and Grossman (9) or after that of Barker *et al.* (10). No significant difference was found between the values for protein-bound and those for total iodine in thyroid tissue.

Another aliquot of the thyroid homogenate (25 to 50 mg.) was subjected to hydrolysis at 37° C. by adding either 4 mg. of crystallized trypsin (Worthington) or 10 mg. of pancreatin (Armour). Digestion with trypsin was allowed to proceed at pH 8.5 (phosphate buffer) for ninety-six hours and digestion with pancreatin at pH 8 (NH_4OH/NH_4Cl

buffer) for forty-eight hours. An additional 2 mg. of trypsin or 5 mg. of pancreatin was added each twenty-four hours. When the I^{131} content was low, the hydrolyzed material was extracted three times with 3 volumes of 0.1 *N* HCl-n-butanol. The combined extracts were brought back to pH 8 by adding *N* sodium hydroxide, and were evaporated to dryness in a vacuum ($<40°$ C.). The residue was dissolved in 0.2 ml. of n-butanol /NH_4OH(95:5).

The thyroid extracts were applied to Whatman No. 1 paper strips with monoiodotyrosine (MIT), diiodotyrosine (DIT), triiodothyronine (T_3), and thyroxine (T_4) as carriers. Descending chromatography was performed in both systems: butanol-acetic acid-water (78:5:17), and iso-amyl-alcohol saturated with ammonia 6*N*. After staining, the labeled iodine of the chromatograms was measured, centimeter by centimeter, with the use of an automatically counting recording strip scanner (Electrophysique, Brussels).

The butanol-acetic acid chromatograms were used to estimate the distribution of I^{131} between MIT and DIT, and the iso-amyl-alcohol-ammonia records were used for T_3 and T_4. The results were expressed as a percentage of the total radioactivity of the strip. The fraction of the thyroid material that remained unhydrolyzed was found to be about 5 per cent after digestion with trypsin and 3 per cent after digestion with pancreatin.

Analysis of blood iodine

Protein-bound I^{131} (PBI^{131}) was determined after precipitation of plasma with ice-cold 10 per cent trichloracetic acid. The precipitate was washed twice with 1 per cent trichloracetic acid and the radioactivity was counted with a well-type scintillation counter; results were expressed as a percentage of the administered I^{131} dose per liter of plasma.

Assays of plasma PBI^{127} and chromatographic studies of the plasma butanol extracts were performed by techniques similar to those used for the thyroid gland.

Calculations

Thyroid weight was calculated by either of 2 methods: 1) by adding to the weight of the removed tissue, the estimated weight of the tissue remaining "in situ" (obtained from comparison of the two pieces by careful palpation during the surgical procedure); or 2) by using the ratio between the thyroidal I^{131} uptake immediately before operation and the I^{131} content of the thyroid homogenate per Gm. of tissue, both expressed as a percentage of the administered dose.

Thyroid organic pool was defined as the product of the iodine concentration (μg. per Gm.) in the thyroid homogenate and the weight of the thyroid (Gm.).

Specific activities of PBI in plasma and thyroid

The secretion rate of the thyroid hormone was indirectly obtained from the labeling rate of the extrathyroidal organic iodine pool after administration of I^{131}. In this attempt, the specific activity of the PBI in plasma was compared with the specific activity of the iodine in the thyroid. The specific activities are expressed as the percentage of the administered dose of I^{131} per μg. of organic iodine. In a two-compartment system (thyroidal and extrathyroidal organic iodine pools), the ratio between the specific activities of plasma and thyroidal organic iodine progressively increases and equals unity at one point in time (3). After this time the ratio may be assumed to approximate unity constantly, if the rate of thyroidal release is small in comparison with the rate of hormonal degradation (3).

RESULTS

Quantitative data on stable and labeled iodine in plasma and thyroid

Plasma. The mean value for plasma PBI^{127} concentration in the 14 euthyroid goitrous patients was 3.8 ± 1.9 μg. per 100 ml. As shown in Table 1, the corresponding levels of plasma PBI^{131} distinctly fell into two different ranges. In the first group of 8 patients ("low PBI^{131} group"), the mean value at twenty-four hours was 0.05 ± 0.03 per cent and at forty-eight hours 0.10 ± 0.05 per cent of the administered dose of I^{131}. Corresponding values in the 6 patients of the "high PBI^{131}" group were respectively 0.44 ± 0.11 per cent and 0.85 ± 0.44 per cent.

TABLE 1. RADIOACTIVE AND STABLE IODINE PATTERN IN THE PLASMA AND THYROID GLANDS OF 14 GOITROUS PATIENTS FROM THE UELE ENDEMIC AREA

Group	Patient No.	Age & sex	Plasma				Thyroid					Interval between I^{131} and operation (days)
			PBI^{127} (μg./100 ml.)	PBI^{131} (% dose/L)			Weight (Gm.)	I^{127}		I^{131} uptake (% dose)		
				24 hrs.	48 hrs.	operation day		conc. (μg./Gm. tissue)	total amount (mg.)	24 hrs.	operation day	
"Low plasma PBI^{131}"	181	25 F	2.4	0.06	0.18	0.26	68	146	9.9	—	—	3
	182	35 M	3.6	0.07	0.12	0.25	85	70	6.0	—	—	3
	183	32 F	1.4	0.04	0.08	0.10	170	97	16.5	—	—	3
	018	45 F	5.1	0.01	—	0.04	—	104	—	67	69	5
	024	30 F	3.9	0.08	—	0.08	104	80	8.6	82	82	5
	027	35 M	—	0.02	—	—	92	—	—	76	78	7
	148	42 M	2.7	0.05	0.05	0.16	182	50	9.1	82	76	7
	149	30 F	6.0	0.05	0.09	0.15	215	35	7.5	86	82	7
"High plasma PBI^{131}"	014	35 F	4.8	0.42	—	0.85	120	120	14.4	78	63	3
	184	38 F	3.7	0.45	1.39	2.02	155	33	5.1	—	—	3
	041	30 F	1.6	0.66	0.98	1.10	54	62	3.4	64	50	5
	028	14 F	7.6	0.37	—	1.32	—	46	—	68	59	7
	015	25 F	2.9	0.32	0.38	0.51	154	79	12.2	95	75	10
	147	28 F	2.9	0.43	0.63	0.72	87	90	7.8	78	73	10

Thyroid. The total iodine content of the thyroid was calculated in 11 patients; the results varied from 3.4 to 16.5 mg. (mean, 10.0 ± 4.1 mg.). The concentration of iodine per Gm. of homogenized gland ranged from 33 to 146 μg. (mean, 79 ± 33 μg.). In the 5 euthyroid Belgian patients the concentration of iodine was 346 ± 86 μg. per Gm. of tissue.

Detailed data concerning the thyroidal uptake of I^{131} in these patients have been previously reported (8). Table 1 shows the uptake values at twenty-four hours (64 to 95 per cent of the administered dose), or from two to nine days later, immediately before the removal of the gland.

Comparison of the two series of determinations in the "low plasma PBI^{131}" patients did not reveal any modification of the thyroidal I^{131} content during the time of observation.

In the "high plasma PBI^{131}" group, on the contrary, there was a partial discharge of the thyroidal radioiodine, ranging from 5 to 20 per cent (mean, 13 per cent) of the administered dose; values for thyroidal I^{131} half-life

were respectively 17.6, 13.5 and 25.8 days in Patients No. 015, No. 028 and No. 147.

Specific activity of organic iodine in plasma and thyroid

The present calculations were performed on the basis of the assumption accepted by Riggs (11), Stanbury *et al.* (3) and Berson and Yalow (12) of a single iodine compartment in the thyroid gland. According to this concept, radioiodine uniformly labels the thyroidal iodine stores which participate as a whole in the hormonal secretion process.

On the other hand, it has been postulated that all the iodine (I^{127} and I^{131}) present in the gland is organically bound. The lack of radioactive iodide observed in thyroid chromatograms and the identity of both organic and total iodine chemical measurements are in agreement with this assumption.

TABLE 2. SPECIFIC ACTIVITIES* OF THE ORGANIC IODINE IN THE THYROID AND THE PLASMA AFTER ADMINISTRATION OF I^{131}

Group	Patient No.	Days after admin. of I^{131}	Thyroid ($\times 10^{-2}$)	Plasma† ($\times 10^{-2}$)	Ratio $\frac{\text{plasma SA}}{\text{thyroid SA}}$
"Low plasma PBI^{131}"	182	3	0.81	0.42	0.52
	018	5	0.14	0.06	0.43
	024	5	0.97	0.20	0.21
	148	7	1.07	0.25	0.23
	149	7	0.81	0.42	0.52
Mean and S.D.			0.76 ±0.36	0.27 ±0.15	
"High plasma PBI^{131}"	014	3	0.54	1.78	3.03
	184	3	1.40	5.45	3.89
	041	5	1.66	6.86	4.13
	028	7	0.26	1.73	6.65
	015	10	0.79	1.78	2.25
	147	10	1.00	2.48	2.48
Mean and S.D.			0.94 ±0.52	3.35 ±2.23	

* Expressed as the % of the administered I^{131} dose per μg. of iodine.
† The plasma sample was withdrawn immediately before the operation.

Table 2 shows the values for the specific activities of the organic iodine in the thyroid gland and in a blood sample withdrawn immediately before the removal of the gland. Both are expressed as a percentage of the administered radioiodine per μg. of PBI.

In the plasma, the mean of the PBI specific activities in the "high PBI^{131}" group was twelve times higher than the mean in the "low PBI^{131}" group. For the thyroid glands, no statistical difference could be detected between both groups.

The ratios of the iodine specific activities in the plasma and in the thyroid gland are listed in Table 2. In the "low PBI^{131}" group all values were less than unity. For the 2 patients operated on seven days after administration of I^{131}, the ratios were respectively 0.2 and 0.5. On the other hand, in the "high PBI^{131}" patients, the ratio was always more than unity, the values ranging from 2.2 to 6.7.

Chromatographic studies of the thyroid gland

Iodinated amino acids. The labeled iodinated amino-acid content of the thyroid was investigated by chromatographic analysis in 13 Uele goi-

TABLE 3. DISTRIBUTION OF I^{131} AMONG THE LABELED IODINATED AMINO ACIDS IN THE THYROID GLAND OF GOITROUS PATIENTS FROM THE UELE ENDEMIC AREA

Type of hydrolysis	Patient No.	Days after I^{131}	Distribution of I^{131} (%)				Ratio $\frac{MIT^{131}}{DIT^{131}}$
			MIT	DIT	T_3	T_4	
Trypsin	018	5	47	15	—*	—*	3.0
	024	5	50	19	—	—	2.6
	041	5	36	22	—	—	1.6
	027	7	54	17	—	—	3.2
	028	7	52	21	—	—	2.5
	148	7	43	16	—	—	2.7
	015	10	30	19	—	—	1.6
	147	10	40	22	—	—	1.8
	149	10	64	24	—	—	2.7
Pancreatin	181	3	46	31	0	traces	1.5
	182	3	35	36	0	9	1.0
	183	3	54	21	0	traces	2.6
	184	3	35	28	3	9	1.3

* No labeled iodothyronine was found in the thyroid glands subjected to trypsin hydrolysis. These observations were not used because of the presence of an artefact (see text).

trous patients. MIT^{131} and DIT^{131} were present in all the glands and in some cases $T_3{}^{131}$ and $T_4{}^{131}$ were present also. Butanol-acetic acid chromatography of trypsin-hydrolyzed material showed the existence of an unidentified radioactive spot; this labeled compound migrated between DIT^{131} and $T_4{}^{131}$, from which it was often difficult to separate. Further studies have shown that this compound[1] was an artefact due to an unhydrolyzed peptide containing some $T_4{}^{131}$. Therefore the results obtained after trypsin digestion were not taken into account in demonstrating the presence of iodothyronines in the gland.

The distribution of I^{131} in the different labeled iodinated amino acids is shown in Table 3.

[1] After elution and further chromatography in butanol-acetic acid, this labeled compound migrated with the same Rf. Submitted to pancreatin hydrolysis, it liberated some $T_4{}^{131}$ and small amounts of DIT^{131} and other unidentified labeled compounds.

Iodotyrosines. In all but 1 of the thyroid glands examined, there was more labeled MIT than labeled DIT. After trypsin hydrolysis, in 9 glands the MIT^{131}/DIT^{131} ratio ranged from 1.6 to 3.6 (mean, 2.4 ± 0.6). After pancreatin digestion, in 4 glands the ratio ranged from 1.0 to 2.6 (mean, 1.6 ± 0.7).

The MIT^{131}/DIT^{131} ratios in normal thyroid fragments (pancreatin hydrolysis) removed from 3 control Belgian patients forty-eight hours after administration of I^{131} were respectively 0.6, 0.7 and 1.1.

Iodothyronines. In only 2 of the 4 pancreatin-treated glands was the presence of labeled iodothyronines unequivocal. In both, T_4 accounted for 9 per cent of the radioactivity; small quantities of T_3 (3 per cent) were found in one of them.

Chromatographic studies of plasma

Chromatographic studies of the thyroid hormones in blood were possible only in the patients of the "high plasma PBI^{131}" group. T_4 but no T_3 was found in all these subjects.

Histologic findings

Diffuse parenchymatous hyperplasia with various degrees of stromal edema was observed in 2 glands. Localized parenchymatous hyperplasia coexisting with large colloid-filled vesicles was noted in 9 glands. There were no signs of thyroiditis.

DISCUSSION AND CONCLUSIONS

It has been shown by Stanbury *et al.* (3) that in the Mendoza endemic goiter area (South America) the mechanism for adaptation of the thyroid gland to iodine deficiency chiefly consists of an increased extraction rate of blood iodine. In other goitrous areas, high thyroidal I^{131} uptakes have been found to be associated with an accelerated release of labeled hormone, a high conversion ratio and a high level of blood PBI^{131}. The similarity of these findings with the pattern observed in hyperthyroidism led Roche *et al.* (6) to postulate a faster iodine turnover in iodine-deficient goiter. The results of the present investigations are not in accordance with this view.

Thyroidal secretion

In spite of its variability, the mean iodine content of the thyroid in the Uele goiter area lies in the range observed by Berson and Yalow (12) in normal and hyperthyroid patients. On the other hand, the I^{131} content of the Uele gland is about twice that observed in the glands of euthyroid patients with a normal iodine supply (13).

In the first group of patients, the blood PBI^{131} values were in the range observed in euthyroid persons with an adequate iodine supply (14). In these patients isotopic equilibrium between the blood PBI level and the thyroidal iodine content was far from being reached even seven days after administration of I^{131}. Since isotopic equilibrium is reached before that time in normal subjects (12), this observation suggests a diminished rate of thyroid secretion.

In the second group of patients (high PBI^{131} values), the specific activity of organic iodine was two to seven times higher in the blood than in the removed thyroid. Either of two explanations could account for these results: (*a*) At the time of operation the greater part of the thyroidal I^{131} was already discharged from the gland; this did not seem to be the case, as the thyroidal specific activity was in the same range as in the first group and the major part of the accumulated I^{131} remained in the gland. (*b*) The labeled hormone secreted in the first few hours after administration of I^{131} had a much higher specific activity than the iodine remaining in the gland. This implies that radioiodine is not homogeneously distributed amongst the thyroid iodine stores but enters into two or more functionally distinct compartments with different turnover times. A similar hypothesis was proposed by Stanbury *et al.* (3) to account for the results of I^{131} turnover calculations performed in some cases of Mendoza goiter. The I^{131} distribution studies performed by Triantophyllidis (15) on men and rats have also been interpreted on the basis of the lack of homogeneity between thyroidal iodine pools.

In the present investigations, the secretion of iodine into a pool with a fast turnover could account for the high level of plasma PBI^{131}. It should be pointed out, however, that since there is no information about the amounts of stable iodine involved in such a pool, its quantitative validity in hormonal secretion cannot be established. As suggested by the low level of plasma PBI^{127} observed in some of these patients, this compartment with a rapid turnover might involve only a small fraction of the total thyroid iodine stores.

If the occurrence in the thyroid of a multi-compartmented iodine distribution should be confirmed, the usual calculations for the dynamics of thyroid secretion (3, 4, 11, 12, 16) may become questionable in certain conditions, since all these methods are based on the assumption of a single iodine pool in the thyroid.

Thyroidal hormonogenesis

The distribution of labeled iodinated amino acids in Uele goiters is different from that observed by Braasch *et al.* (17) and by Pitt-Rivers *et al.* (18) in the thyroid glands of normal and hyperthyroid patients.

In the Uele goiters there was a definite decrease in the labeled diiodotyrosine and iodothyronine fractions of thyroglobulin. The absence of modification in the relative amounts of I^{131} between MIT and DIT from the third to the tenth day after administration of I^{131} suggests that the distribution is similar to that of the corresponding stable compounds.

Similar abnormalities of iodine distribution in the thyroid gland have been reported in sporadic nontoxic goiter (18, 19) and in thyroid carcinoma (20). A high MIT^{131}/DIT^{131} ratio and a decreased iodothyronine fraction have also been observed in propylthiouracil-treated rats (21, 22); these findings suggest that in addition to its blocking action on iodination of thyroglobulin, this antithyroid drug inhibits the conversion of MIT to DIT (22) and the coupling of the iodotyrosines into iodothyronines (21). The question may then arise of the possible action of a goitrogen in the development of Uele endemic goiter. No epidemiologic evidence has been presented in favor of such a mechanism (23).

The modifications of the distribution of labeled iodinated amino acids in Uele thyroids can be explained on the basis of the severe iodine deficiency observed in this country. It has been shown by several workers (1, 2, 24, 25) that, in rats, a prolonged iodine-deficient diet induces similar modifications of the thyroid content.

The MIT^{131}/DIT^{131} ratio, as shown by Bois and Larsson (25), reflects the degree of iodination of thyroglobulin. These authors found that in rats pretreated with propylthiouracil, the administration of progressively increasing amounts of iodide induced a parallel increase of the labeled diiodotyrosine fraction. During the *in vitro* iodination of tyrosine the relative proportions of MIT and DIT formed depend upon the quantity of iodide present (23). With reference to the iodothyronine fraction, Querido, Schut and Terpstra found only small amounts of stable and of labeled T_4 and T_3 in the thyroids of rats fed during six months with a Remington diet (2).

The lowered concentration of T_4^{131} in Uele goiters cannot be explained (at least in the patients of the "low PBI^{131}" group) by the output of this hormone from the gland. In these patients, indeed, the amounts of labeled hormone discharged until the time of operation represented only a very small fraction of the thyroidal I^{131} stores.

The results of chromatographic analysis thus indicate that in the Uele goiter the rate of transfer of I^{131} into the more heavily iodinated compounds (DIT and the iodothyronines) is slower than in normal glands. The reason for this slowing down is unknown. However, it is noteworthy that the concentration of iodine in these goitrous glands amounts to only approximately one-fourth of that in the control glands. This marked decrease in the iodine available for the various steps of thyroidal hormonogenesis probably constitutes the primary alteration of iodine metabolism in Uele goiter.

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Letters to the Editor

REAPPEARANCE OF MENSTRUATION AND GONADOTROPINS WITH TREATMENT OF SECONDARY THYROID AND ADRENOCORTICAL FAILURE IN SHEEHAN'S SYNDROME

To the Editor:

Secondary gonadal failure usually predominates over thyroidal and adrenal failure as the earliest and most persistent manifestation of anterior pituitary insufficiency from any cause. Exceptions to this generalization are known. Very occasionally, patients with postpartum necrosis of the pituitary exhibit some spontaneous recovery of pituitary function (1). However, if amenorrhea and other signs of failure of the peripheral glands persist, it is generally considered to be an irreversible phenomenon due to irreparable damage to the gonadotropic and other secretions of the pituitary. The observation that gonadotropic and gonadal function recovered in a patient with apparent complete pituitary failure is of some interest.

The patient was a 29-year-old housewife who had an uneventful pregnancy and delivery at 21 years of age. At age 25 ,she had another uneventful pregnancy but suffered a severe postpartum hemorrhage with peripheral vascular collapse. Three blood transfusions and additional supportive measures were employed to manage the emergency. No lactation occurred in the postpartum period and her breasts became atrophic. Thereafter, she became completely amenorrheic, and all of the typical signs and symptoms of anterior pituitary failure developed (pale, hairless, dry, fine skin; complete loss of sexual hair; easy fatigue; hypotension; intolerance to cold; normochromic anemia; and atrophy of the uterus and vaginal mucosa). When first seen at age 27 (two years after the postpartum hemorrhage), the basal metabolic rate was −38 per cent of normal, the serum protein-bound iodine (PBI) level was 0.7 μg. per 100 ml., and urinary 17-ketosteroid excretion was 1.1 mg. per twenty-four hours.

She was treated with 25 mg. of cortisone and 120 mg. of desiccated thyroid per day, with rather dramatic general improvement. Much to everyone's surprise, after four months of this therapy she began menstruating at regular four-week intervals. Menstrual periods were accompanied by her usual moderate degree of premenstrual tension and dysmenorrhea, and

generally lasted four days. A PBI determination during this interval was 4.0 μg. per 100 ml. (normal). After menstruating regularly for eleven months, the cortisone and thyroid were tapered off, and stopped for three months. Almost all of her earlier symptoms gradually recurred and she again became amenorrheic. The PBI level fell to 0.5 μg. per 100 ml., and 17-ketosteroid excretion was low (1.7 mg. per twenty-four hours). Apparently the recovery of general pituitary function had not been spontaneous. Cortisone and desiccated thyroid were restarted, and within two

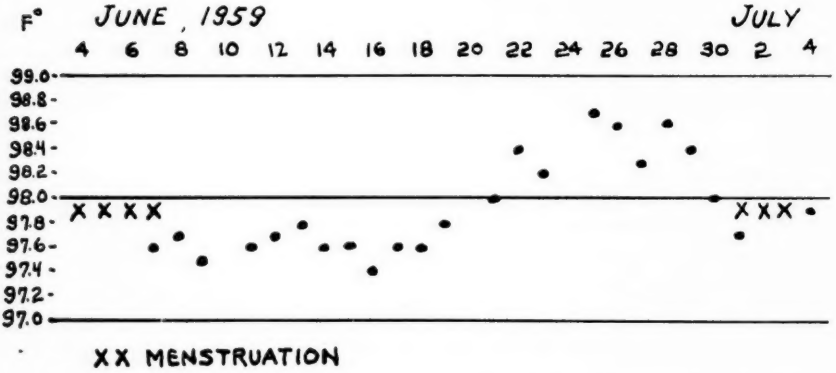

FIG. 1. An example of the basal temperatures during one menstrual cycle. These were obtained approximately two months prior to the above assay for pituitary gonadotropin.

months regular menses recurred. At mid-cycle, a 24-hour urine contained 12 rat units of pituitary gonadotropin (kindly assayed by Dr. Alexander Albert).[1] Her basal temperature indicated ovulation (Fig. 1).

One year later, medication was again discontinued. Two months thereafter, no gonadotropin could be detected in a 24-hour urine at mid-cycle (<6.6 mouse units). Replacement therapy was restarted and the patient has been in good general health since. Because she and her husband have employed contraceptives, it is not known if she is fertile.

In this case potentially good residual pituitary gonadotropic function was secondarily impaired by thyroidal and/or adrenocortical failure, and

[1] By this method normally menstruating, premenopausal women excrete either undetectable amounts or up to 25 rat units of pituitary gonadotropin at mid-cycle. It is of interest that gonadotropin was detected in the case reported here.

treatment of these secondary deficiencies allowed recovery of some pituitary activity.

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URINARY EXCRETION OF STABLE IODINE IN A SMALL GROUP OF ISOLATED VENEZUELAN INDIANS

An elevated thyroidal I^{131} uptake without apparent goiter has been demonstrated previously in a small group of isolated Indians belonging to the Maquiritare and the Guaharibo tribes from the Alto Ventuari region in the Amazon territory of Venezuela (1). In February 1960, during a more recent expedition, 24-hour urine collections were obtained from 18 Indians of the Maquiritare tribe, 9 of whom had undergone radioiodine uptake tests in February 1959. Water from the Ventuari River was obtained from six different sites. The study was performed in Kakuri, but most of the Indians came from Korobal, a small village about 15 miles downstream; a few came from near the Salto Mono, 10–15 miles upstream. The Indians use river water almost exclusively for drinking. In the previous study in the same region (1) the thyroid gland in a total of 89 Indians had been examined; 72 were non-palpable or barely palpable, 14 were palpable but apparently normal, 2 were slightly enlarged on palpation, and 1 (in a girl about 12 years of age) was visibly enlarged upon close scrutiny and contained a firm, round nodule, about 3 cm. in diameter in the left lobe.

METHODS

The 24-hour urines from the 18 Indians were collected in wide-mouth glass jars, thoroughly rinsed with double-distilled water. Accurately measured aliquots were kept in tightly stoppered polyethylene bottles, with a few drops of toluol added. The Ventuari River water samples were obtained in polyethylene bottles and kept without preservative. Urine and water samples were flown to Caracas on the last day of collection, and kept in an ice box until they were sent to Mendoza, by plane, seven days later.

Control urine samples were collected from laboratory and Institute personnel, and control drinking water samples from the Caracas area. These were treated similarly and flown to Mendoza.

Iodine determinations were performed by Barker's method (2) as modified by Carr *et al.* (3).

RESULTS

The results may be seen in Tables 1 and 2. In Table 1 the Subject numbers below 100 are those asigned to the Indians studied in the 1959 expedition; the Subject numbers above 100 are those assigned to the Indians not

TABLE 1. FINDINGS IN ALTO VENTUARI INDIANS OF THE MAQUIRITARE TRIBE

Subject		Clinical status of thyroid†	Urine (24-hr. sample)		Thyroidal I^{131} uptake (1959 expedition) (% of dose)
No.*	Sex & estimated age		Vol. (ml.)	Iodine (μg.)	
1	M 45	N.P.	2480	47.7	66.6
7	M 22	N.P.	2650	34.5	69.2
16	M 27	P	2190	16.4	88.0
21	M 28	N.P.	1500	14.2	—
27	M 18	P	2450	33.0	76.8
29	F 27	P	740	13.0	53.2
32	M 20	D.E.	3600	17.4	—
34	M 15	D.E.	1120	27.5	91.2
41	F 40	N.P.	1970	13.3	—
43	F 32	N.P.	495	53.5	—
49	M 16	P	1020	27.5	71.6
55	M 20	N.P.	1100	11.0	—
77	M 37	N.P.	1900	8.2	76.2
79	M 18	N.P.	2300	17.5	79.6
101	F 30	?	1750	19.7	—
102	F 27	?	790	8.3	—
103	F 30	?	1000	11.3	—
104	F 32	?	950	7.6	—

* See text for explanation of numbers.

† N.P. = not palpable; P = barely palpable; D.E.—slightly and diffusely enlarged, but not visible.

studied previously and in whom the thyroid was not palpated. In the latter subjects, the thyroid was not visibly enlarged to an observer without medical experience.

Urinary iodine excretion was low—on the average 21.2 μg. per day, with a mode between 10 and 20 μg. In 1951, before iodized salt began to be used, Stanbury *et al.* (4) found that iodine excretion in Mendoza, Argentina, was on the average 23.6 μg. per day. In Venezuela, De Venanzi *et al.* (5) found a value of 17.8 μg. ±1.86 in 13 persons without goiter, 11.6 μg. ±1.2 in 20 subjects with diffuse goiter, and 11.1 μg. ±2.76 in subjects with nodular goiter, all of them coming from La Mesa de Esnujaque, an endemic goiter area in the Venezuelan Andes. In a similar area (Bailadores) Roche *et al.* (6) found an average excretion of 22.5 μg. per day in

12 subjects, with range from 9.0 to 40.0 μg. Thus the values for the Alto Ventuari region are similar to those for the highly endemic Andean regions. In comparison, the control values for the Caracas region, where there is no endemic goiter and where the thyroidal radioiodine uptake is normal (7), ranged from 31.6 to 127.9 μg. per day, with an average of 67.7 μg.

The iodine content of drinking water was unusually low in Alto Ventuari (Table 2); in none of the samples did it reach 1.0 μg. per liter. In Mendoza, the iodine content of drinking water ranges between 1.28 and 13.07 μg. per liter (8). In La Mesa in the Venezuelan Andes, in samples obtained

TABLE 2. IODINE CONTENT OF DRINKING WATER

Region	Site	Iodine content (μg./liter)
Alto Ventuari	Kakuri (drinking site)	0.2
	Kakuri (mid-stream)	0.2
	Korobal (drinking site)	0.6
	Korobal (mid-stream)	0.3
	Salto Mono	0.3
	"Pueblo Arriba"	0.2
Caracas	La Mariposa	17.5
	Vista Alegre	4.0
	Altamira	29.0
	Altos de Pipe	19.8

from three different sites, De Venanzi *et al.* (5) obtained values of 1.36, 1.50, and 4.22 μg. per liter.

The very low iodine values for drinking water were taken to indicate a low environmental iodine content. That there were sources of alimentary iodine other than drinking water is obvious, as witnessed by the significant, although low, urinary excretion of iodine. Further studies, including iodine analyses of staple foods and water, repeated thyroidal I^{131} uptakes, and urinary iodine analyses in this and similar regions are admittedly required. The low iodine content of drinking water and of human urine in the Alto Ventuari region, however, constitutes further evidence that a condition of iodine deficiency prevails in that area. One of the results is an elevated thyroidal I^{131} uptake, without endemic goiter. What constitutes endemic goiter is largely a matter of definition. The same barely palpable gland will be called normal if the patient comes from a nongoitrous area, and goitrous if the patient comes from an endemic region. In any case, the Alto Ventuari region cannot be called a goitrous area on the basis of the data available. In the group of Indians presented here, of the 14 patients whose thyroid was examined, only 7 had a nonpalpable gland. In the other 7 subjects, 5 glands were barely palpable and 2 were diffusely enlarged. Even if the 7 latter patients are called goitrous, they are in no way what one

would expect to see in other areas with similarly low iodine intakes and high thyroidal iodine accumulation.

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THYROTROPIN IN THE SERUM OF MOTHER AND FETUS

To the Editor:

A sensitive bioassay for thyrotropin (TSH) has been developed by McKenzie (1) from the technique of Adams and Purves (2). The present study was undertaken to determine the amount of TSH in maternal and cord blood, with the use of this assay.

Blood was taken from the mother during labor and from the umbilical cord immediately after delivery in a series of 10 normal births.

RESULTS

A sensitivity of 0.02 mU with an injection volume of 0.5 ml., and an average index of precision (λ) of 0.247 were established. Thyrotropin was detected in the sera of 16 of 20 euthyroid subjects (0.084–0.175 mU per ml.). The levels of TSH in maternal and cord blood at delivery were in the normal range and the difference was not significant at the 5 per cent level (Table 1).

Table 1. Concentration of the thyrotropin in sera from pregnant women and from the cord at delivery

Patient, number and name	Week of pregnancy	Thyrotropin in serum (mU/ml.)	
		Maternal blood	Cord blood
1—S.W.	40	0.150	0.113
2—H.S.	40	0.175	0.094
3—Y.Y.	40	0.119	0.086
4—O.H.	39	0.099	0.112
5—K.K.	40	0.118	0.085
6—Y.S.	39	0.104	0.112
7—T.A.	39	0.111	0.142
8—S.T.	37	0.120	0.150
9—F.S.	40	0.125	0.125
10—K.T.	39	0.120	0.100
	Mean	0.124*	0.112*
	S.D.	±0.0247	±0.0185

* Difference is not significant at the 5% level.

DISCUSSION

Döderlein (3) stated that TSH in guinea pigs passes through the placenta and stimulates the fetal thyroids. More recent work in the guinea pig, however, has indicated that TSH does not enter the fetal circulation through the placenta (4).

In the present study, the difference in the levels of TSH between maternal and cord blood in the human was not significant, and the concen-

tration of TSH in both was within the normal range. If TSH does not cross the human placenta, then the fetal hypothalamus and pituitary maintain a concentration of TSH similar to that in the adult.

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The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street,

Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

Thirteenth Postgraduate Assembly in Endocrinology and Metabolism

UNDER THE CO-SPONSORSHIP OF THE ENDOCRINE SOCIETY
AND THE NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND
October 2–6, 1961

A comprehensive review of clinical endocrine problems and current research activity in these areas will be presented. For further information, write to: Dr. Roy Hertz, National Institutes of Health, Building 10, Bethesda 14, Maryland. The fee will be $100.00 for physicians, with a reduction to $30.00 for Residents and Fellows. Enrollment limited to 100.

Block reservations are being held until September 15 at the following nearby motels: Bethesdan Motor Hotel, 7740 Wisconsin Ave., Bethesda, and Intown Motor Hotel, Wisconsin Ave. and Bradley Blvd., Bethesda. Registrants and faculty members must complete their own arrangements for housing by direct correspondence with these or other nearby motels. Reservations should be completed at least one month in advance.

PROGRAM

CHAIRMEN: Dr. Roy Hertz and Dr. F. C. Bartter

Monday Morning, Oct. 2

8:00– 9:00 Registration

PITUITARY AND HYPOTHALAMUS. Chairman: *Dr. Charles W. Lloyd*

9:00– 9:35 Experimental Studies in Neuro-Endocrine Relationships. *Dr. Evelyn Anderson*

9:35–10:00 Chemistry of Pituitary Hormones. *Dr. Peter Condliffe*

10:10–10:45 Bioassay and Function of Pituitary Hormones. *Dr. Robert Bates*

10:45–11:00 Intermission

11:00–11:40 Clinical Disorders of Anterior Pituitary. *Dr. Griff T. Ross*

11:40–12:20 Clinical Disorders of Mid-Brain and Posterior Pituitary. *Dr. Charles W. Lloyd*

12:20– 1:00 Endocrine Effects of Hypophysectomy and Stalk Section in Man. *Dr. M. B. Lipsett*

1:00– 2:00 Lunch

Monday Afternoon

2:00– 5:00 Case Presentations, Panel and Discussion: Disorders of Mid-Brain, Posterior Pituitary and Anterior Pituitary. *Drs. Lipsett, Lloyd, Bartter, Ross, McCullagh*

5:30– 6:30 Cash Bar Cocktail Party and Reception—Navy Officer's Club

Tuesday Morning, Oct. 3

PARATHYROID DISORDERS. Chairman: *Dr. F. C. Bartter*

9:00– 9:30 Metabolic Effects of Parathyroid Hormone. *Dr. F. C. Bartter*
9:30–10:00 Hyperparathyroidism. *Dr. F. R. Keating.*
10:00–10:30 Hypoparathyroidism. *Dr. F. Engel*

10:30–10:45 Intermission

10:45–11:15 Osteoporosis. *Dr. Donald Whedon*
11:15–11:45 Metabolic Bone Disease (other than Osteoporosis). *Dr. F. C. Bartter*
11:45–12:15 Calcium Metabolism in Sarcoidosis. *Dr. Norman Bell*
12:15– 1:00 Panel and Case Presentations. *Drs. Bartter, Keating, Engel, Whedon*
1:00– 2:00 Lunch

Tuesday Afternoon

DISORDERS OF CARBOHYDRATE METABOLISM. Chairman: *Dr. E. Perry McCullagh*

2:00– 2:30 Carbohydrate Metabolism. *Dr. DeWitt Stetten*
2:30– 3:00 Insulin—Mechanisms and Assays. *Dr. J. Field*
3:00– 3:30 Clinical Uses and Mode of Action of Synthetic Hypoglycemic Agents. *Dr. F. Lukens*
3:30– 5:00 Panel—Cases. *Drs. McCullagh, Lukens, Field, Engel*

Wednesday Morning, Oct. 4

PROBLEMS IN REPRODUCTION AND GONADAL FUNCTION. Chairman: *Dr. Roy Hertz*

9:00– 9:30 The Interpretation of Testicular Biopsies and Sex Chromatin Pattern Tests. *Dr. W. O. Nelson*
9:30–10:00 Metabolism Effects of Androgenic and Progestational Steroids. *Dr. Richard Landau*
10:00–10:30 Clinical Syndromes of Testicular Malformation and Dysfunction. *Dr. E. P. McCullagh*

10:30–10:45 Intermission

10:45–11:30 Sexual Precocity. *Dr. Roy Hertz*
11:30–12:15 Hirsutism, Stein-Leventhal and Related Syndromes. *Dr. M. B. Lipsett*
12:15– 1:00 Hormonal Relations Between Mother and Fetus in Pregnancy. *Dr. Claude Migeon*
1:00– 2:00 Lunch

Wednesday Afternoon

2:00– 2:30 Gonadal Dysgenesis in Phenotypic Females. *Dr. Henry Turner*
2:30– 3:00 Secondary Amenorrhoea. *Dr. Charles W. Lloyd*
3:00– 3:30 Fertility and Sterility. *Dr. Celso-Ramon Garcia*

3:30– 5:00 Panel—Cases. *Drs. McCullagh, Turner, Garcia, Migeon, Hertz, Lloyd*

6:00 Cocktails (Cash Bar): Dinner for Registrants and Faculty, Navy Officer's Club

Thursday Morning, Oct. 25

THYROID DISORDERS. Chairman: *Dr. Rulon Rawson*

9:00– 9:30 Experimental Thyroid Tumors. *Dr. S. Wollman*
9:30–10:30 Thyroid Cancer. *Dr. Rulon Rawson*

10:30–10:45 Intermission

10:45–11:15 Thyroxin Analogues—Their Properties and Uses. *Dr. S. Asper*
11:15–12:15 Evaluation of Medical Therapy of Thyroid Disease. *Dr. F. R. Keating*
12:15– 1:00 Thyroxin-Binding Proteins in Iodine Metabolism. *Dr. J. Robbins*

1:00– 2:00 Lunch

Thursday Afternoon

THYROID DISORDERS (continued). Chairman: *Dr. B. Dobyns*

2:00– 3:00 Indications and Procedures for Thyroid Surgery. *Dr. B. Dobyns*
3:00– 3:30 Autoimmunity Studies in Thyroid Disease. *Dr. J. Robbins*
3:30– 5:00 Cases and Panel. *Drs. Rawson, Dobyns, Asper, Keating, Robbins*

Friday Morning, Oct. 6

ADRENAL DISORDERS. Chairman: *Dr. F. C. Bartter*

9:00– 9:30 Adrenal Physiology. *Dr. C. Migeon*
9:30–10:00 Tests of Adrenal Function. *Dr. B. Kliman*
10:00–10:30 Adrenal Insufficiency. *Dr. J. Beck*

10:30–10:45 Intermission

10 45–11:15 Hyperadrenocorticism with Respect to Hydrocortisone. *Dr. F. Engel*
11:15–11:45 Hyperadrenocorticism with Respect to Aldosterone. *Dr. F. C. Bartter*
11:45–12:15 Hyperadrenocorticism with Respect to Androgen. *Dr. C. Migeon*
12:15– 1:00 Panel and Cases. *Dr. Bell, Engel, Migeon, Kliman, Beck and Bartter*

1:00– 2:00 Lunch

Friday Afternoon

2:00– 3:00 Endocrine Aspects of the Cancer Problem. *Dr. Roy Hertz*
3:00– 3:45 Lipid Metabolism. *Dr. D. Fredrickson*

FACULTY

EVELYN ANDERSON, M.D., Chief, Section on Endocrinology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

SAMUEL P. ASPER, JR., M.D. The Johns Hopkins Hospital, Baltimore, Maryland

FREDERICK C. BARTTER, M.D., Chief, Clinical Endocrinology Branch, National Heart Institute, Bethesda, Maryland

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J. C. BECK, M.D., Royal Victoria Hospital, Montreal, Canada

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BROWN DOBYNS, M.D., Ph.D., Associate Professor of Surgery, Western Reserve University School of Medicine and Assistant Director of Surgery, Metropolitan General Hospital, Cleveland, Ohio

FRANK L. ENGEL, M.D., Professor of Medicine and Associate Professor of Physiology; Director, Division of Endocrinology, Duke University Medical Center, Durham, North Carolina

D. S. FREDRICKSON, M.D., Clinical Director, National Heart Institute, Bethesda, Maryland

CELSO-RAMON GARCIA, M.D., The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

ROY HERTZ, M.D., Ph.D., Chief, Endocrinology Branch, National Cancer Institute, Bethesda, Maryland

F. R. KEATING, JR., M.D., Mayo Clinic, Rochester, Minnesota

BERNARD KLIMAN, M. D., Clinical Endocrinology Branch, National Heart Institute, Bethesda, Md.

RICHARD L. LANDAU, M.D., Professor of Medicine, University of Chicago School of Medicine, Chicago, Illinois

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E. PERRY MCCULLAGH, M.D., Head, Section of Endocrinology and Metabolism, Cleveland Clinic, Cleveland, Ohio

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G. DONALD WHEDON, M.D., Chief, Metabolic Diseases Branch, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

SEYMOUR H. WOLLMAN, Ph.D., Laboratory of Physiology, National Cancer Institute, Bethesda, Maryland

The American Thyroid Association

The next Annual Meeting of the American Thyroid Association will be held in the Hotel Roosevelt, New Orleans, Louisiana, *May 9–12, 1962.*

At its 1961 Annual Meeting, the American Goiter Association became the American Thyroid Association. Theodore Winship, M.D., is now the Secretary and the business office is 430 North Michigan Avenue, Chicago 11, Illinois.

The Mexican Society of Nutrition and Endocrinology

The Second Annual Meeting of the Mexican Society of Nutrition and Endocrinology will be held at San Jose Purua, Michoacan, Mexico, *November 24–25, 1961.* Short communications, limited to 15 minutes will be presented in Spanish or English. Abstracts not exceeding 350 words should be submitted by August 31, 1961. Based on these abstracts, papers will be selected for oral presentation.

The full text and figures of the accepted papers will be reproduced and made available to each participant at the beginning of the meetings. Each complete text must therefore be in the hands of the Secretary by October 8, 1961.

Additional information regarding the meeting and hotel accommodations can be obtained from the Secretary: Dr. Carlos Gual, Hospital de Enfermedades de la Nutricion, Calle Dr. Jimenez 261, Mexico 7, D.F.

New York University Post-Graduate Medical School

Course in Gynecological Endocrinology

A practical, didactic and clinical presentation with emphasis on the therapeutic management of endocrine disorders in the female, including a discussion of the diagnosis and management of intersex will be offered. Endocrine therapy for menstrual abnormalities and ovulatory defects will be presented. Adrenocortical, thyroid, ovarian and pituitary abnormalities will be emphasized. Practical diagnostic tests will be described and demonstrated. The therapeutic use of the newer progestational, gonadal and corticoid steroids will be discussed, as well as available pituitary preparations. Some pertinent aspects of male and pediatric endocrinology will be included, as will the diagnostic importance of chromosomal analysis. Five-day, full-time course, *October 9 through October 13, 1961*. Tuition: $140.00. Given under the direction of Dr. Herbert S. Kupperman. For further information write the Office of the Associate Dean, New York University Post-Graduate Medical School, 550 First Avenue, New York 16, N. Y.

Urology Award

The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300, and third prize $200) for essays on the result of some clinical or laboratory research in urology. Competition is limited to urologists who have been graduated not more than ten years, and to hospital internes and residents doing clinical or laboratory research work in urology. Animal research is not necessary. The first-prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Bellevue-Stratford Hotel, Philadelphia, Pennsylvania, May 14–17, 1962. For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore 1, Maryland. Essays must be in his hands *before November 15, 1961*.